ELSEVIER

Journal of Chromatography A, 679 (1994) 277–284

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatography of antihistamines using post-column tris(2,2′-bipyridine) ruthenium(III) chemiluminescence detection

John A. Holeman, Neil D. Danielson*

Department of Chemistry, Miami University, Oxford, OH 45056, USA

First received 24 November 1993; revised manuscript received 8 June 1994

Abstract

The separation and detection of five antihistamine drugs commonly found within over-the-counter allergy and cold pharmaceutical products was performed by HPLC with chemiluminescence (CL) detection. Comparable detection limits at 5–10 pmol were found for the antihistamines by both UV at 214 nm and tris(2,2′-bipyridine) ruthenium(III) CL. However, urine samples were found not to generate as large an unretained peak by CL detection as compared to those peaks by UV detection at 214 and 254 nm. For example, the pheniramine peak representing 0.15 μg/ml was almost totally obscured at 214 nm. Quantitative results received for three antihistamine commercial samples ranged from 4 to 8% error in accuracy when an internal standard was used to compensate for short term detector drift.

1. Introduction

Chemiluminescence (CL) has been applied to a wide variety of compounds and ions as a means of detection for chromatography [1]. Recently, several studies have utilized tris(2,2′-bipyridine) ruthenium(III) [$Ru(bpy)_3^{3+}$] as an CL reagent that oxidizes various organic amines. This reagent will in general react best with tertiary, then secondary, and finally primary alkyl amines [2,3]. Studies have utilized this reagent to quantitate antibiotic compounds like erythromycin and clindomycin which both have a reactive tertiary amine [4,5]. Recently, diuretic hydrothiazide compounds such as hydrochlorothiazide and hydroflumethiazide were found to chemiluminesce even at the 1–2 pmol level [6]. Amino acids were found to vary greatly in reactivity depending upon the R group. In particular, proline [7] and tryptophan [8] which have the more reactive secondary amine substituent can be detected in the pmole range. Detection limits for primary alkyl amino acids can be improved under basic conditions [9,10]. Dansylated amino acids having a tertiary amine group attached to an aromatic ring can be determined after HPLC separation at the 2 pmol level by $Ru(bpy)_3^{3+}$ CL [11]. Oxidation of $Ru(bpy)_3^{2+}$ immobilized on a Nafion-coated electrode in a flow cell mounted in front of a photomultiplier tube (PMT) permits reuse of $Ru(bpy)_3^{3+}$ but analyte detection limits are only in the nmole range [12]. Electrochemical

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00548-N

generation of $Ru(bpy)_3^{3+}$ in solution on-line at either a Pt [13] or glassy carbon [14] electrode has been demonstrated but the limited concentration of oxidizing agent formed could deleteriously affect detection limits.

Antihistamines are found within a wide variety of over-the-counter cold and allergy medication products. Potential side effects caused by antihistamines when accompanied with alcohol, hypnotic agents, and antianxiety drugs are drowsiness and depression of the central nervous system [15]. Antihistamines are also sometimes used in combination with other drugs to treat adverse side affects. For instant, diphenhydramine is sometimes used to treat acute dystonic symptoms brought about by antipsychotic drugs used to treat patients with Parkinson disease [16].

In this paper, the determination of five antihistamines brompheniramine, chlorpheniramine, pheniramine, diphenhydramine, and pyrilamine (structures shown in Fig. 1) by $Ru(bpy)_3^{3+}$ CL after HPLC separation is described. The quantitative analysis of three cold medication products and the potential of $Ru(bpy)_3^{3+}$ CL for the determination of drugs in urine samples is provided.

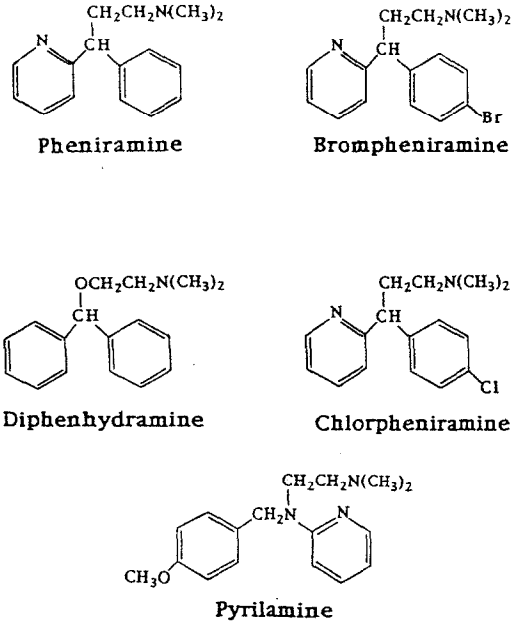

Fig. 1. Structures of five tertiary antihistamines.

2. Experimental

2.1. Equipment

A Waters Model 510 HPLC pump (Milford, MA, USA) was used to transport the sample under chromatographic and flow injection analysis (FIA) modes. The $Ru(bpy)_3^{2+}$ solution was oxidized to $Ru(bpy)_3^{3+}$ with a Princeton Applied Research (Princeton, NJ, USA) Model 174A polarographic analyzer using a platinum gauze working electrode, a platinum wire auxiliary electrode, and a silver wire reference electrode. The reagent reservoir was continuously bubbled with helium gas for 30 min before applying a +950-mV potential across the electrodes. A magnetic stir bar was also used during the purging, reagent oxidation, and analysis processes. A Rheodyne Model 7010 injector (Cotati, CA, USA) with a 20-μl loop was used throughout all the studies. The $Ru(bpy)_3^{3+}$ oxidizing reagent was transported by an Alita peristaltic pump (Medina, WA, USA) that utilized 1.0 mm (0.040 inch) I.D. Tygon pump tubing. The reagent flow stream connection between a diagonally cut PTFE tube (0.5 mm I.D. × 1.5 mm O.D.) inserted in the Tygon pump tube to the fused-silica capillary utilized a male/male union in which a Kel-F ferrule and graphite ferrule sealed the PTFE and capillary lines, respectively.

A modified Waters 420-AC fluorometer was used to detect the CL reaction within the 8-μl flow cell. A stainless-steel tube (O.D. = 1.6 mm; I.D. = 1.0 mm) leading out of the top of the 8-μl flow cell to a stainless-steel tee was fitted with a fused-silica capillary (I.D. = 317 μm; O.D. = 427 μm) coaxially with enough excess space available to allow waste to exit between the tubing walls and out through one branch of the tee. Introduction of the $Ru(bpy)_3^{3+}$ occurred through this capillary that was fixed in position by a PTFE ferrule in the stainless-steel tee. The polyamide coating of the capillary within the quartz flow

cell was removed by burning the capillary tip with a match and wiping away the carbon coating. No modification was made on the inlet portion of the fluorometer. The excitation source of the fluorometer was removed but the position of the flow cell, optics, and PMT remained unchanged. A Hamamatsu PMT Model R928YP2547 with maximum sensitivity in the visible light region was substituted. Characterization of this flow cell design which ensures the CL light signal (about 1 s in duration) occurs in front of the PMT has been carried out previously [5]. The data were collected with a Shimadzu Chromatopac C-R6A integrator (Kyoto, Japan). UV analysis was performed on an Applied Biosystems (Ramsey, NJ, USA) Model 757 variable wavelength absorbance detector.

The chromatographic column used to separate the antihistamines was a 150×4.6 mm I.D. Asahipak ODP-50 C_{18} column purchased from Keystone Scientific (Bellefonte, PA, USA). Flow-rates used for all experiments were 0.80 ml/min and 0.28 ml/min for the chromatographic and reagent streams, respectively. These flow-rates are in the optimum range found previously in another study [5].

2.2. Reagents

A 1 m*M* tris(2,2'-bipyridine) ruthenium(II) hexachloride solution (salt obtained from Aldrich in Milwaukee, WI, USA) was prepared with either a 0.05 *M* acetate-buffered supporting electrolyte (pH 5.5) or 0.05 *M* sodium sulfate. Although a 2 m*M* $Ru(bpy)_3^{3+}$ solution when oxidized provided a somewhat greater CL signal, the 1 m*M* solution was used to conserve reagent.

Buffer solutions were prepared at concentrations of 0.2 *M* at pH values of 5.5, 7.0, 8.0, 9.0, and 10.0. A phosphate salt along with the borate salt was required for buffers pH 9.0 and 10.0 to maintain a consistent molarity to other buffers. An acetate salt was used for pH 5.5 and a phosphate salt for 7.0 and 8.0 buffers. The acetonitrile was supplied from Burdick and Jackson, (Muskegon, MI, USA). The antihistamines brompheniramine, chlorpheniramine, diphenhydramine, pheniramine, and pyrilamine were purchased through Sigma (St. Louis, MO, USA). In-house water was purified with a Barnstead E-Pure system (Dubuque, IA, USA).

Pharmaceutical samples Benadryl (diphenhydramine), Chlor-Trimeton (chlorpheniramine) tablets, and Dimetapp (brompheniramine) grape flavor cough syrup were analyzed. The antihistamine tables were prepared by crushing and grinding the tablet contents within a polystyrene weighing dish. The crushed contents were transferred into a volumetric flask with 100 ml of water along with 30–40 ml of acetonitrile to assist in dissolving the antihistamines. These solutions were diluted to 250 or 500 ml with water. A 10- to 20-ml portion of each of these tablet solutions was then centrifuged to remove any undissolved solids. A known quantity of the centrifuge supernatant was quantitatively diluted to a calculated concentration determined by the manufacturer's cited active ingredient amount. All standards and samples were spiked with N,N-dimethylbenzylamine as an internal standard.

3. Results and discussion

The optimum CL pH values for all five antihistamines were determined by FIA methodology. A comparison of the change in chemiluminescence intensity with pH is shown in Fig. 2. Pheniramine, chlorpheniramine, and brompheniramine are found to be the most reactive at pH 9.0. This alkaline pH optimum agrees with previous work involving amino acids [9] and supports the general trend of greater reactivity for compounds having unprotonated amine functional groups. The like reactivity of pheniramine, chlorpheniramine, and brompheniramine is reasonable since all three have similar structures. The tertiary nitrogen not the pyridine nitrogen is reasonable for the CL reaction. The diphenhydramine reactivity loss may be due to the ether functional group within the alkyl chain between the tertiary amine and aromatic rings. A similar trend involving polar side chains has been proposed to explain the different $Ru(bpy)_3^{3+}$ CL responses for amino acids [10]. Pyrilamine is less

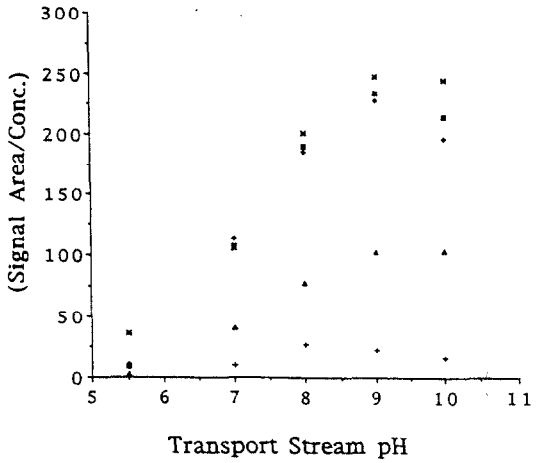

Fig. 2. CL response vs. pH for five antihistamines. Sample concentrations (M): (■) chlorpheniramine $6.3 \cdot 10^{-6}$, (◆) brompheniramine $4.4 \cdot 10^{-6}$, (▲) diphenhydramine $5.9 \cdot 10^{-6}$, (×) pheniramine $3.9 \cdot 10^{-6}$, (+) pyrilamine $4.3 \cdot 10^{-6}$.

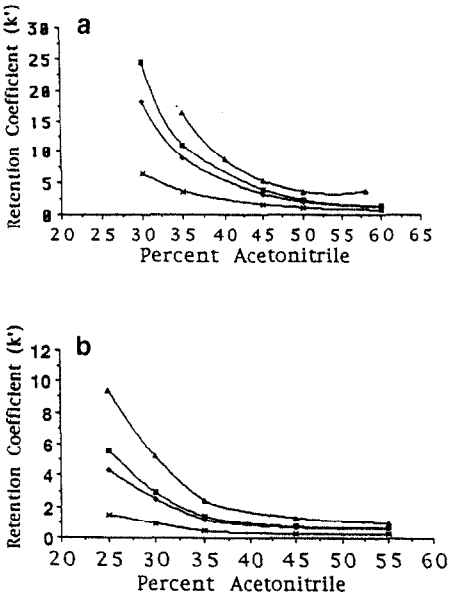

Fig. 3. Retention factor curves for changes in % acetonitrile for four antihistamines at (a) pH 9.0 and (b) pH 7.0. ■ = Brompheniramine, ◆ = chlorpheniramine, ▲ = diphenhydramine and × = pheniramine.

reactive when compared to the other four antihistamines (about a factor of 4 and 9 less compared to diphenhydramine and pheniramine, respectively). A CL reactivity study was done on two model compounds, 2-dimethylaminopyridine and 2-benzylaminopyridine. The latter compound was ten times less reactive than pyrilamine indicating the benzyl group may be quenching. However this compound is a secondary amine and is expected to generate a weaker CL signal. 2-Dimethylaminopyridine was found to be seven times more reactive than pyrilamine even though pyrilamine has 2 tertiary nitrogens and this model compound has only one. This lends support back to the idea that this electron withdrawing benzyl group is probably quenching the pyrilamine CL signal. We also know that nitro groups can quench the CL signal since N,N-dimethylaniline can be detected at the 5 pmol level at pH 3 but N,N-dimethyl-3-nitroaniline does not react.

3.1. Chromatography

Reversed-phase separation of these antihistamines using a polymeric C_{18} column under basic and neutral conditions requires a moderate quantity of an organic solvent. Previous studies showed acetonitrile caused less quenching of the $Ru(bpy)_3^{3+}$ CL reaction as compared to methanol [5]. Fig. 3 shows the retention factors of four antihistamines under neutral and basic conditions as a function of percent acetonitrile. Under neutral pH conditions the antihistamines become more protonated and k' values are about a factor of 7 less. The optimum acetonitrile–water ratios when separating these four antihistamines were 47:53 at pH 9.0 and 35:65 at pH 7.0. A fifth compound pyrilamine is retained similarly to brompheniramine but all five drugs can be separated upon reduction of the acetonitrile content in the mobile phase under neutral pH conditions (Fig. 4).

3.2. Detection limits

Good detection limits have been cited for these antihistamines. Detection limits for chlorpheniramine of about 0.5 pmol within 1-ml samples of plasma or saliva after extraction with diethylether and preconcentration were reported at a 254 nm wavelength [17]. Optimum UV wavelength conditions have been reported for

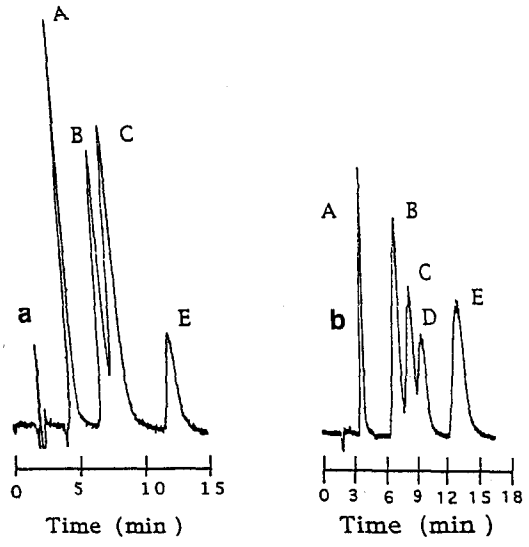

Fig. 4. Chromatograms for the separation of (a) four and (b) five antihistamines. (a) Acetonitrile–0.05 *M* borate buffer, pH 9.0 (47:53). (b) Acetonitrile–0.2 *M* phosphate buffer, pH 7.0 (27:73). A = Pheniramine, B = chlorpheniramine, C = brompheniramine, D = pyrilamine, E = diphenhydramine.

brompheniramine and pheniramine at 254 nm, and 214 nm for diphenhydramine, but no detection limits were provided [18]. A fluorometric method involving the derivatization of chlorpheniramine with benzyl chloroformate was able to determine 0.1 ng/ml within whole blood samples [19]. Although this derivatization procedure is general for tertiary amines, it only works with dry sample residues.

A detection limit comparison at pH 9 was made between the CL detector and the UV detector (Table 1). Comparable results (5–10 pmol) are achieved for the CL detector and the UV detector at 214 nm for pheniramine and brompheniramine. Chlorpheniramine and diphenhydramine can be detected somewhat better at 214 nm than by CL. However, the CL response shows a 2–3 times improvement in detection limits for brompheniramine, chlorpheniramine, and pheniramine when compared to UV at 254 nm. Diphenhydramine at 254 nm exhibits a dramatic fall off in detectability by one order of magnitude compared to the CL detector. The pyrilamine CL detection limit is about one order of magnitude less responsive than that of the other antihistamines. The antihistamine CL detection limits shown in Table 1 are slightly better than the values reported for dipropylamine (16 pmol) but not as low as that for tripropylamine (0.2 pmol). This tends to indicate that tertiary amines with large bulky aromatic functional groups (brompheniramine, chlorpheniramine, and pheniramine) react similarly to secondary amines. Using a 100-μl injection loop, concentration CL detection limits can be reduced to 0.01–0.03 μg/ml for all four compounds in Table 1.

3.3. Quantitative analysis

To alleviate CL detector drift observed after 2–3 h, internal standards were chosen for their strong reactivity to the oxidizing reagent and ability to be separated from most of the antihistamines. One internal standard (N,N-dimethylbenzylamine) was added to the standards and samples of diphenhydramine, chlorpheniramine, and brompheniramine to compensate for the CL drift. *tert.*-Butylbenzylamine could be used as an alternative internal standard for pheniramine although it happens to coelute near

Table 1
Detection limit comparison between UV detector and a chemiluminescence detector

Detector	Pheniramine	Brompheniramine	Chlorpheniramine	Diphenhydramine
Chemiluminescence	0.09 μg/ml (8 pmol)	0.14 μg/ml (9 pmol)	0.14 μg/ml (10 pmol)	0.21 μg/ml (16 pmol)
UV 214 nm	0.07 μg/ml (6 pmol)	0.13 μg/ml (8 pmol)	0.07 μg/ml (5 pmol)	0.10 μg/ml (8 pmol)
UV 254 nm	0.20 μg/ml (17 pmol)	0.41 μg/ml (26 pmol)	0.29 μg/ml (21 pmol)	2.4 μg/ml (290 pmol)

Table 2
Linear least square regression data

Compound	$Y = A + BX$	Correlation coefficients (No. data points)	Linear range studied (μg/ml)
Brompheniramine	$A = 1.1 \cdot 10^{-1} \pm 3.2 \cdot 10^{-2}$	0.999	0.5–22
	$B = 2.7 \cdot 10^{-1} \pm 2.9 \cdot 10^{-2}$	(12)	
Chlorpheniramine	$A = 1.4 \cdot 10^{-1} \pm 5.6 \cdot 10^{-2}$	0.997	0.3–15
	$B = 3.0 \cdot 10^{-1} \pm 6.7 \cdot 10^{-3}$	(15)	
Diphenhydramine	$A = 2.5 \cdot 10^{-2} \pm 3.2 \cdot 10^{-2}$	0.993	0.5–7.5
	$B = 1.8 \cdot 10^{-1} \pm 7.6 \cdot 10^{-3}$	(10)	

Y = Peak area sample/peak area internal standard. $X = \mu$g/ml.

chlorpheniramine and brompheniramine. Linear least squares regression analysis data are provided in Table 2. Reproducibility of duplicate or triplicate data points is about 2–10% R.S.D. With the internal standard, percent errors from the label values of 5–6% for diphenhydramine and 4–8% for chlorpheniramine and brompheniramine in three pharmaceutical products are calculated.

3.4. Matrix effects

Chromatograms of the three pharmaceutical samples with detection at 214 nm and CL were compared. Only single peaks due to diphenhydramine are observed in the Benadryl chromatograms regardless of detection method. An unretained peak which did not interfere with the chlorpheniramine peak is noted for both Chlor-Trimeton chromatograms. The Dimetapp sample matrix did cause some interference for the brompheniramine analyte as indicated by the shoulder peak (Fig. 5A). Several other unknown components are observed within the Dimetapp chromatogram. When this chromatogram is compared to the Dimetapp CL chromatogram in Fig. 5B, no interferences can be seen for brompheniramine.

We also compared chromatographic results between an UV detector and CL detector for three antihistamine-spiked urine samples. The urine matrix generated a large unretained absorbance response for UV wavelengths at both 214 and 254 nm that did not come down to baseline until about 12 min. The unretained signal caused by this urine sample was smaller for the CL detector coming down to baseline in about 5 min. It could be attributed possibly to amino acids found in urine [20] such as histidine and tryptophan which have secondary amine groups as well as other amines that give strong signals at alkaline pH. Both urea and uric acid did not react with $Ru(bpy)_3^{3+}$ to generate CL. Previously we have reported that carbonyl groups next to the nitrogen atom are electron withdrawing preventing facile oxidation of the analyte to a radical by $Ru(bpy)_3^{3+}$ [7]. At 254 and 214 nm, the unretained urine components

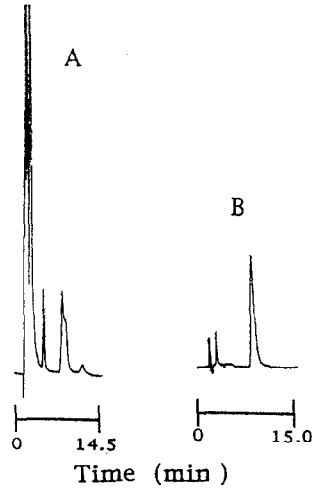

Fig. 5. Dimetapp (4.0 μg/ml brompheniramine 8.4 min) sample chromatograms with detection at (A) 214 nm and (B) CL. Mobile phase: acetonitrile–0.01 M borate buffer, pH = 9.0 (47:53). The peak at 5.1 min in chromatogram A is the internal standard, N,N-dimethylbenzylamine.

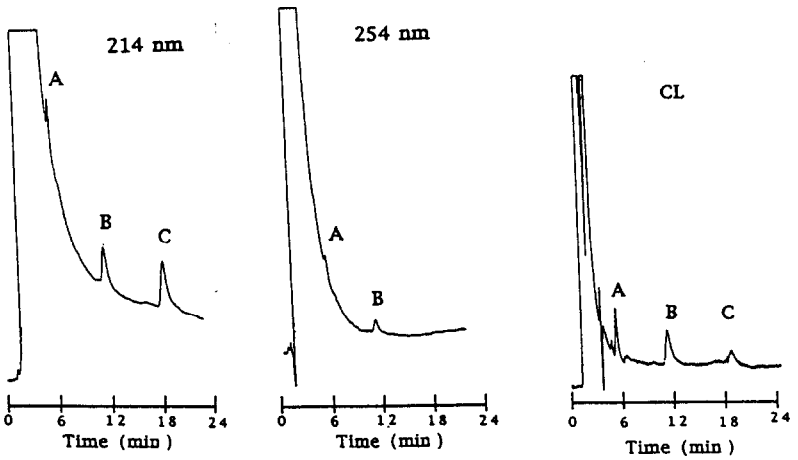

Fig. 6. Chromatograms of an undiluted urine sample spiked with 0.15 μg/ml pheniramine (A), 0.26 μg/ml brompheniramine (B), and 0.29 μg/ml diphenhydramine (C) taken with UV (214 nm), UV (254 nm), and CL detection. Mobile phase: acetonitrile–0.025 *M* borate buffer, pH 9.0 (40:60). Flow-rate: 0.8 ml/min. Injection size: 100 μl.

such as uric acid greatly interfere with the determination of 1.1 μg/ml of pheniramine which shows up as a shoulder peak (data not shown). Baseline resolution of pheniramine from the urine matrix peak is possible using the CL detector. This UV urine matrix problem is still evident for 5 μg/ml brompheniramine which elutes off later than the pheniramine analyte (data not shown). Fig. 6 shows a comparison of UV 214 nm, UV 254 nm, and CL chromatograms for a urine sample spiked at low levels (0.1–0.3 μg/ml) of pheniramine, brompheniramine, and diphenhydramine. These concentrations are more typical of those found in urine for these antihistamines. No sample preconcentration using a column or extraction was done. For the chromatograms detected at 214 and 254 nm, pheniramine is almost completely obscured by the urine matrix. Diphenhydramine cannot be detected at this level at 254 nm. Complete resolution and detection of all three drugs on a non-sloping baseline was possible using the CL detector.

Acknowledgement

Financial support through a NIH AREA grant is gratefully appreciated.

References

[1] T.A. Nieman, in J.W. Birks (Editor), *Chemiluminescence and Photochemical Reaction Detection in Chromatography*, VCH, New York, 1989, Ch. 4.
[2] J.B. Noffsinger and N.D. Danielson, *Anal. Chem.*, 59 (1987) 865.
[3] J.B. Noffsinger and N.D. Danielson, *J. Chromatogr.*, 387 (1987) 520.
[4] N.D. Danielson, L. He, J.B. Noffsinger and L. Trelli, *J. Pharm. Biomed. Anal.*, 7 (1989) 1281.
[5] M.A. Targove and N.D. Danielson, *J. Chromatogr. Sci.*, 28 (1990) 505.
[6] J.A. Holeman and N.D. Danielson, *Anal. Chim. Acta*, 277 (1993) 55–60.
[7] L. He, K.A. Cox and N.D. Danielson, *Anal. Lett.*, 23 (1990) 195.
[8] K. Uchikura and M. Kirisawa, *Chem Lett.*, (1991) 1373.
[9] S.N. Brune and D.R. Bobbitt, *Talanta*, 38 (1991) 419.
[10] S.N. Brune and D.R. Bobbitt, *Anal. Chem.*, 64 (1992) 166.
[11] W.-Y. Lee and T.A. Nieman, *Anal. Chem.*, 64 (1994) 111.
[12] T.M. Downey and T.A. Nieman, *Anal. Chem.*, 64 (1992) 261.
[13] K. Uchikura and M. Kirisawa, *Anal. Sci.*, 7 (1991) 803.
[14] W.A. Jackson and D.R. Bobbitt, *Anal. Chim. Acta*, 285 (1994) 309.
[15] T.H. Wiser, T.D. Guberski and M.B. Wiener, in M.B. Wiener (Editor), *Clinical Pharmacology and Therapeutics in Nursing*, McGraw-Hill, New York, 1979, Ch. 21.
[16] D.T. Witiak, in A. Burger (Editor), *Medicinal Chemistry, Part II*, Wiley-Interscience, New York, 3rd Ed., 1970, Ch. 65.

[17] N.K. Athanikar, G.W. Peng, R.L. Nation, S.M. Huang and W.L. Chiou, *J. Chromatogr.*, 162 (1979) 367–376.
[18] A.S. Sidhu, J.M. Kennedy and S. Deeble, *J. Chromatogr.*, 391 (1987) 233–242.
[19] Y. Miyamoto, *J. Chromatogr.*, 420 (1987) 63–72.
[20] H.C Damm and J.W. King (Editors), *Handbook of Clinical Laboratory Data*, CRC, Cleveland, OH, 1965, p. 49.