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Determination of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid in urine by automated pre-column derivatization with 2,3-naphthalene dicarboxyaldehyde and high-performance liquid chromatography with fluorescence detection

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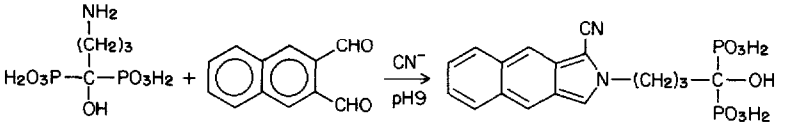
ABSTRACT

A sensitive (5 ng/ml) method for the determination of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid in human urine is described The procedure includes (1) the isolation of the drug from urine by co-precipitation of its calcium salt with endogenous phosphates in the presence of base, (2) a solid-phase anion-exchange sample clean-up and (3) automated pre-column derivatization of the primary amino group with 2,3-naphthalene dicarboxyaldehyde–cyanide reagent followed by fluorescence detection of the N-substituted cyanobenz[*f*]isoindole derivative The derivative of the drug was synthesized and its spectral and fluorescence properties were evaluated The fluorescence quantum efficiency was determined to be 0 82 in the mobile phase used for the assay The derivative is also capable of accepting energy in an oxalate ester–hydrogen peroxide chemiluminescence system

INTRODUCTION

The geminal bisphosphonate, 4-amino-1-hydroxybutane-1,1-bisphosphonic acid (I, Fig. 1), is a potent inhibitor of bone resorption and is being evaluated for the treatment of a variety of bone diseases such as Paget's disease, hypercalcemia of malignancy and osteoporosis.



I II

Fig 1 Structures of I and its product (II) of derivatization with 2,3-napthalene dialdehyde–cyanide reagent

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Several methods have been described for the compounds related to I in pharmaceutical dosage forms [1–5], where isolation of the drug from the matrix and high sensitivity of detection were usually not required. Few analytical methods have been described for the determination of bisphosphonates related to I in biological fluids [6,7]. In one of these procedures, based on decomposition of the P–C–P bond with ultraviolet (UV) light and spectrophotometric determination of the resulting inorganic phosphate, the limit of quantification, defined as the lowest concentration that can be assayed with a relative standard deviation of ±10%, was about 500 ng/ml in 5 ml of urine. In a more recent example of quantitation of disodium 3-amino-1-hydroxypropylidenebisphosphonate (APD) in dog urine, the method was based on derivatization with fluorescamine and fluorescence detection of the derivative [8]. The limit of quantitation was about 200 ng/ml using a 2-ml sample.

Also recently, a very elaborate and tedious method for quantification of APD in urine and plasma based on high-performance ion chromatography and post-column oxidation of bisphosphonate to orthophosphate was described [9]. The limited validation data presented indicated a limit of quantification in urine of 450 ng/ml in 1 ml of urine. Fels *et al.* [10] have also reported an assay in urine and plasma for (4-chlorophenyl)thiomethylene bisphosphonic acid. The assay is based on ion-pair reversed-phase high-performance liquid chromatography (HPLC) with UV detection since this compound possesses a UV-absorbing chromophore (4-chlorophenyl) for direct detection. The quantification limit in urine and plasma was 50 ng/ml.

None of the methods described above were sufficiently sensitive to be considered as potentially useful for the determination of I in support of clinical pharmacokinetic studies. Therefore, a novel methodology based on the isolation of the drug from urine by a combined calcium salt co-precipitation–solid-phase extraction procedure and by automated pre-column derivatization of the primary amino group has been developed. A highly fluorescent N-substituted cyanobenz-[*f*]isoindole (CBI) derivative (II, Fig. 1) of I is formed with the 2,3-naphthalene dicarboxyaldehyde (NDA)–cyanide (CN^-) reagent [11–14]. The limit of reliable quantification for the new method (5 ng/ml using a 5-ml urine sample) was found to be far superior to all other procedures presently available for determination of structurally similar bisphosphonates.

EXPERIMENTAL

Materials

HPLC-grade methanol, tetrahydrofuran (THF) and ethyl acetate, and analytical-grade sodium acetate, sodium hydroxide, calcium chloride, sodium mono- and dihydrogenphosphate, sodium pyrophosphate, trichloroacetic acid (TCA), potassium cyanide, sodium acetate, hydrochloric acid and hydrogen peroxide (30%) were from Fisher (Fair Lawn, NJ, U.S.A.). The sodium citrate and ethyle-

nediaminetetraacetic acid (EDTA) were from Sigma (St. Louis, MO, U.S.A.). 2,4,6-Trichlorophenyl oxalate (TCPO) originated from Fluka (Ronkokoma, NY, U.S.A.). The monosodium salt trihydrate of compound I was obtained from Merck Sharp & Dohme (Rahway, NJ, U.S.A.). The NDA was purchased from Molecular Probes (Eugene, OR, U.S.A.). Deionized water was prepared using a Milli-Q reagent water system (Millipore, Milford, MA, U.S.A.).

Instrumentation

A Varian Vista Model 5500 HPLC system with a Varian 9090 autosampler (Walnut Creek, CA, U.S.A.) was used for all analyses. As a detector, the Perkin-Elmer spectrofluorometer (Model 650-10S, Norwalk, CT, U.S.A.) equipped with an HPLC flow cell was utilized. The Spectroflow 783 UV detector (Kratos, Ramsey, NY, U.S.A.) was also utilized in the exploratory part of the study. The detector output signals were interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, U.S.A.). Absorption spectra were obtained using a diode array spectrophotometer (HP 8452) and also directly from HPLC runs using a photo-diode array UV detector (Polychrom 9060, Varian). The nuclear magnetic resonance (NMR) and mass spectra were obtained using a Nicolet NT 360-MHz instrument (General Electric, Fremont, CA, U.S.A.) and a VG ZAB-H (VG Masslab, Stamford, CT, U.S.A.) instrument, respectively.

The analytical column was a Polymer Labs. (Amherst, MA, U.S.A.) polymeric reversed-phase (PLRP-S, 100 Å, 5μm) column (150 mm × 4.6 mm I.D.). Hamilton PRP-1 cartridges (20 mm × 4.6 mm I.D., Reno, NV, U.S.A.) were used as guard columns. For the additional clean-up of the urine extracts, diethylamine (DEA) solid-phase extraction cartridges (3 ml, 500 mg; Analytichem International, Harbor City, CA, U S.A.) were utilized.

Chromatographic conditions

The mobile phase was a mixture of methanol–0.025 *M* sodium citrate–0.025 *M* dihydrogenphosphate, adjusted to pH 8.5 with 10 *M* sodium hydroxide (4:6, v/v) and delivered at a flow-rate of 1.0 ml/min. The effluent from the column was monitored by a fluorescence detector with the excitation and emission wavelengths set at 420 and 490 nm, respectively. All mobile phase components were measured separately, mixed and filtered through a 0.2-mm nylon 66 filter (Rainin Instruments, Woburn, MA, U.S.A.).

Standard solutions

Stock solutions were stored at − 5°C and kept for a period of up to two weeks. Five working standard solutions with concentrations of I equal to 0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml were prepared by the appropriate dilution of a stock standard. Additional stock standards, independent from those used for preparing working standards, were used to make quality control (QC) urine samples spiked at 10 and 80 ng/ml. The QC samples were prepared in 5-ml aliquots of control urine and stored at − 20°C until assayed.

Sample preparation

The standard curve was determined daily by analyzing control human urine (5 ml) spiked with 50 μl of working standards of I. Each solution of standard in urine, placed in a 150 mm × 15 mm glass culture tube, was vortex-mixed followed by the addition of 50 μl of 2.5 *M* calcium chloride. The addition of 50 μl or more of 1 *M* sodium hydroxide, as needed, was used to form a slight white precipitate. After centrifugation the urine was aspirated off and the white pellet was redissolved in 50 μl of 1 *M* hydrochloric acid. Following the addition of 5 ml of water, the calcium salts were precipitated again by the addition of 50 μl of 1 *M* sodium hydroxide. After centrifugation and aspiration of the supernatant, the pellet was reconstituted in 50 μl of 1 *M* hydrochloric acid followed by the addition of 0.01 *M* EDTA (1 ml) and 0 1 *M* sodium acetate buffer pH 4.0 (2 ml). This solution was then applied to a DEA cartridge preconditioned with 3 ml of water. After washing the cartridge with water (3 ml), the drug was eluted with 3 ml of 0.05 *M* sodium citrate and 0.05 *M* sodium phosphate buffer pH 8.5 (1:1, v/v). A part of the sample eluent (250 μl) was then placed in an amber sample vial and 250 μl of 0.1 *M* sodium borate buffer pH 9.1 were added. The vial was capped, vortex-mixed and placed on the autosampler which was programmed to add automatically 10 μl of 0.05 *M* potassium cyanide followed by 10 μl of the stock solution (2 mg/ml) of NDA in methanol. After allowing the reaction to proceed for 15 min, 100 μl of the sample were automatically injected onto the HPLC column.

With minor modifications the above procedure was also applied to the determination of I in plasma. The plasma sample (1 ml) was deproteinized with 1 ml of 10% TCA. After vortex-mixing and centrifugation for 10 min at 5300 *g*, the supernatant was transferred to a second glass tube and 50 μl of 0.1 *M* sodium pyrophosphate in water were added. After the addition of 50 μl of 2.5 *M* calcium chloride, the procedure was the same as for the assay in urine.

Precision, linearity and specificity

The precision of the method was determined by replicate analyses ($n = 5$) of human urine containing I at 5, 10, 25, 50 and 100 ng/ml. The linearity of each standard line was confirmed by plotting the drug concentration *versus* peak height. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard curve. The weight was set to equal the inverse of the variance at each concentration. The specificity of the assay was checked by running blank urine from different sources; endogenous interferences were not observed.

Synthesis of the derivative II

Compound II was synthesized using a procedure similar to that described in ref. 11 with some modifications. To a stirred suspension of NDA (92 mg, 0.5 mmol) in 25 ml of methanol, 25 mg of sodium cyanide (0.5 mmol) was added

followed by 136 mg (0.5 mmol) of I dissolved in 4 ml water. The reaction was performed in a Pyrex flask covered with aluminium foil to prevent photochemical decomposition of the product. After 45 min of stirring at room temperature a red precipitate was recovered by filtration and the solid material was dried under vacuum. The yield of the sodium salt of II was 82% (182 mg). The ^{1}H NMR spectrum was as follows: (^2H_2O, 360 MHz) 8.39 (s, 1H), 8.10 (s, 1H), 8.13 (s, 1H), 7.89 (t, 2H, J = 12Hz), 7.36 (m, 2H), 4.50 (t, 2H, J = 11Hz), 2.42 (m, 2H), 2.02 (m, 2H) (s = singlet, t = triplet, m = multiplet); this accounts for all the protons of the CBI moiety of II and the methylene protons originating from I. The fast atom bombardment (FAB) mass spectrum of II (Xe, in thioglycerol) gave the pseudo-molecular ion peak at *m/e* 425 ($[M + H]^+$, 25%) and the fragment peaks at *m/e* 399 (19%), 383 (28%) and 349 (100%).

Fluorescence quantum efficiency

The absolute fluorescence quantum efficiency (Φ_f) for II was determined relative to quinine sulfate as standard (Φ_f = 0.55 in 0.5 *M* sulfuric acid) [15]. The necessary corrections of the fluorescence spectra and measurements of the relative Φ_f in different solvents were performed using a similar procedure as described earlier [13].

Chemiluminescence (CL) of II in the oxalate ester–hydrogen peroxide system

Hydrogen peroxide in THF (0.1 *M*) and II in acetonitrile ($4 \cdot 10^{-5}$ *M*), and a small volume of base (sodium acetate, 10 μl, final concentration $5 \cdot 10^{-4}$ *M*) were added to the solution containing 2,4,6-TCPO in ethyl acetate ($1 \cdot 10^{-2}$ *M*), in a 1-cm (4 ml) quartz cell in the cell compartment of the spectrofluorometer. The total volume of the solution was 2 ml. With the excitation lamp turned off, the emission was monitored either at fixed wavelength (490 nm) *versus* time after addition of base, or the full spectrum of emission was taken after an initial "burst" of CL light and compared with the fluorescence spectrum of II.

RESULTS AND DISCUSSION

The successful development of the analytical procedure for the routine determination of I in biological fluids at low ng/ml levels required three key steps: the efficient isolation of this highly polar and water-soluble compound from the sample matrix, derivatization to convert this non-chromophoric molecule into a highly absorbing or fluorescing analogue and utilization of some non-conventional chromatography and automation to quantify and separate the highly polar and potentially unstable derivative from the impurities extracted from urine or plasma. Each of these three steps will be addressed here separately.

Isolation of I from urine and plasma

This was accomplished by a modified version of a technique first utilized by

Bisaz *et al.* [6] for the bisphosphonates analogous to I. It was based on co-precipitation of I with naturally occurring phosphates present in urine by the addition of calcium chloride under basic conditions. In order to make the calcium isolation step compatible with derivatization which required basic (pH 9) conditions (*vide infra*) the calcium ions had to be removed from the acidified mixture to assure that I remained in solution as a free acid and not as an insoluble calcium salt at pH 9. Several solid-phase anion-exchange cartridges were evaluated. A weak anion exchanger, a diethylamine (DEA) cartridge, was found to work the best for removing the calcium ions and other urine impurities from I. The presence of the trivalent citrate anions in the eluent (see Experimental) was critical for the effective removal of the anionic form of I from the cartridge. Also, the presence of the citrate ions in the HPLC mobile phase was necessary to decrease the retention time of II and to improve its peak shape.

In the case of plasma, the precipitation of the calcium salt of I from the basified supernatant obtained after deproteinization of plasma with TCA was not effective. The lack of precipitation was probably due to the absence of other co-precipitating phosphates which were present in urine. In order to induce precipitation, sodium pyrophosphate was added to the plasma supernatant before the addition of calcium chloride. Under these conditions, very efficient precipitation and formation of a characteristic pellet was observed.

Choice of derivatizing reagent

There are several derivatizing reagents which could potentially be used for converting compounds containing a primary amino group to fluorescent derivatives [16,17]. Because of our familiarity and previous experience [11,13] with the lately developed NDA–CN^- system and its superiority [12–14] over *o*-phthalaldehyde (OPA)–thiol, dansyl chloride and other reagents commonly used for the determination of primary amino acids and amines, it was decided to examine the NDA–CN^- reagent first for sensitive detection of I. The reaction was rapid and complete at low concentrations and produced the desired, highly fluorescent product II (Fig. 1).

The structure of II has been confirmed by NMR and mass spectroscopy of the synthesized material. Also, a typical absorption spectrum characteristic of the CBI derivatives was obtained with the absorption maxima (λ_{max}) and molar absorption coefficients (ε_{max}) at 442 nm (5400 M^{-1}), 418 (5700) and 252 (55 300). Similarly, the fluorescence emission band typical of the CBI derivatives with two maxima at 458 and 476 nm (uncorrected) was also observed.

The fluorescence quantum efficiency (Φ_f) of II was determined in the mobile phase utilized in the assay. The Φ_f value was measured to be 0.82, the highest ever recorded for a CBI derivative [13].

In order to further evaluate the fluorescent properties of II and compare it with other fluorescent derivatives, the intrinsic fluorescence sensitivity (IFS) factor has been calculated. The IFS factor which provides an empirical comparison

of the sensitivity for a derivative in a standard fluorescence assay [18] is the product of fluorescence quantum efficiency (Φ_f), the molar absorption coefficient (ε_{max}) at the excitation wavelength and the reciprocal of the full width at half-height ($W_{1/2}$) of the emission band. The calculated IFS value of 1.7 for II at λ_{exc} = 418 nm was found to be comparable with those for other CBI derivatives of amines and amino acids [13] which are known to exhibit excellent fluorescence characteristics.

Chromatography and automated derivatization

The high polarity of the eluate II and the need for basic HPLC conditions negated the use of conventional reversed-phase columns such as C_8 or C_{18}. Among various polymeric columns capable of working at elevated pH, the best results were obtained on a column packed with PLRP-S. A sodium citrate–dihydrogenphosphate solution, adjusted to pH 8.5 and mixed with methanol (6:4, v/v) was chosen as an HPLC mobile phase to ensure the derivative stability and to control the charge associated with the phosphonate groups.

The application of this methodology to the analyses of large numbers of clinical samples made automation of at least some parts of the procedure highly desirable. More important, automation of the derivatization step was necessary to eliminate the need for careful control of the reaction kinetics and to eliminate the problem associated with the potential chemical and photochemical instability of II. Instead of a typical precolumn derivatization of many samples and subsequent analyses of these samples over an extended period of time (for example for 20 h), each sample was derivatized at precisely the same time prior to the analysis. This was accomplished utilizing the "Automix" capability of the autosampler. The samples were processed manually to the point of placing aliquots of the eluent from the DEA cartridges with the borate buffer into sample vials. These solutions were stable for up to five days. The autosampler was then programmed to add appropriate volumes of solutions containing CN^- and NDA. The sample solution was allowed to react for 15 min and then injected onto the HPLC column. While the chromatographic run was performed on this derivatized sample, the next sample was derivatized exactly in the same manner as the previous one and injected.

Assay validation

The assay for I was linear in the concentration range 5–100 ng/ml of urine, and the typical equation of the linear regression line was $y = 23665x - 5022$ (using peak heights in mV for calculations). The mean correlation coefficient of six standard curves run over a period of two weeks was 0.9976 [coefficient of variation (C.V.) = 0.21%]. The mean value of the slopes of the daily standard lines were 22967, with a C.V. value of 5.7%. The within-day precision of the assay was less than 10% for all concentrations within the standard curve range (Table I). The accuracy of the assay was 95–106% (Table I).

TABLE I

INTRA-DAY VARIABILLITY AND ACCURACY OF THE ASSAY OF I IN PLASMA

Concentration (ng/ml)	C V (n = 5) (%)	Accuracy[a] (%)
5	3.4	106
10	1.8	96
25	6.7	99
50	5.2	95
100	8.4	103
Mean		100 ± 5

[a] Calculated as (mean observed concentration/nominal concentration) multiplied by 100

The total recovery (efficiency of extraction from urine and derivatization) was determined by comparison of the slopes of the standard lines for spiked standards and the synthesized derivative II injected directly onto the HPLC system. The overall recovery of the drug from extraction and derivatization steps within the whole assay range 5–100 ng/ml was 94 ± 3%.

Inter-day precision, as measured by the concentration of quality control samples over a period of two weeks, was also below 10% (Table II).

Fig. 2 illustrates the chromatograms of II after isolation of I from urine and derivatization. The analyses of various urine blank samples demonstrated the absence of interfering endogenous compounds, confirming the adequate assay specificity.

The preliminary data for the assay of I in plasma were also obtained. The standard line in plasma ($y = 105400x + 19434$ using the peak areas for calculation) in the concentration range 5–50 ng/ml gave a correlation coefficient of 0.9971. A 1-ml volume of plasma was processed here instead of 5 ml of urine. The specificity of the method was established by the analysis of plasma blanks; impurities coeluting with II were not detected.

TABLE II

INTER-DAY VARIABILITY FOR THE ASSAY OF QUALITY CONTROL URINE SAMPLES SPIKED WITH I

Nominal concentration (ng/ml)	Determined concentration[a] (mean ± S.D) (ng/ml)	C.V. (%)
10	9.4 ± 0 6	6.4
80	77 4 ± 6 0	7.8

[a] Mean of seven analyses performed over the period of two weeks

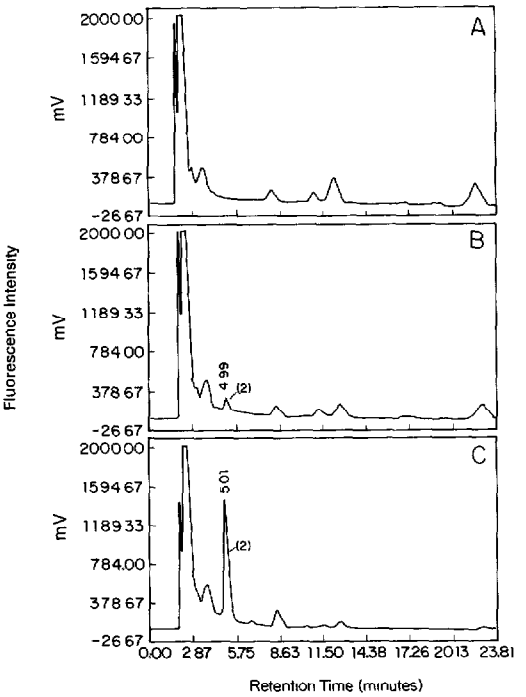

Fig. 2 Representative chromatograms of human urine spiked with I (A) Blank control urine; (B) control urine spiked with 5 ng/ml I, (C) post-dose urine sample of human subject (0–2 h collection period) after oral dosing with 50 mg of I; the concentration of I is equivalent to 44 4 ng/ml

Analysis of samples from human clinical studies

The assay was used for the determination of I in human urine from a pilot clinical study. The representative data are given in Table III.

TABLE III

REPRESENTATIVE CONCENTRATIONS AND AMOUNT OF I EXCRETED IN URINE AFTER ORAL ADMINISTRATION OF 50 mg OF I TO SELECTED FEMALE SUBJECTS

Subject	Pre-dose	0–2 h		2–4 h		4–6 h		6–8 h		8–10 h	
		ng/ml[a]	μg[b]	ng/ml	μg	ng/ml	μg	ng/ml	μg	ng/ml	μg
1	0	18 2	10 9	26.3	6 6	9 2	1 4	–[c]	–[c]	5 8	0 9
2	0	85.4	46.2	54 6	27 3	40 1	11 0	12.3	8 8	–[c]	–[c]
3	0	50.0	16 5	30 0	13 9	25.4	3.1	6 9	2.2	–[c]	–[c]

[a] Concentration over the time interval of urine collection

[b] Calculated by multiplying the volume (ml) of the urine excreted in a given time interval by the concentration (ng/ml)

[c] Concentration in urine below assay's limit of reliable quantification (5 ng/ml).

A very important observation was made concerning the analyses of urine samples from clinical studies. Since the subjects receiving I are often in a hypercalcemic state, the solid calcium salts are often found in these subjects' urine collection vessels. Co-precipitation of I at this point results in loss of drug since only an aliquot of the total urine volume is submitted for analysis. In order to avoid this, the urine must be acidified immediately after collection prior to taking any aliquots and freezing.

Chemiluminescence of II in the oxalate ester–hydrogen peroxide system

Derivative II was found to be a good energy acceptor in a typical oxalate ester–hydrogen peroxide system. The CL spectra closely resembled the fluorescence spectra, indicating that the CL emission orginated from the same singlet excited state. The observation of this emission creates the possibility of developing an assay for the CBI-derivatized I based on HPLC with CL detection [19,20].

CONCLUSION

A sensitive assay in human urine for the determination of I based on solid-phase extraction and automated pre-column derivatization with NDA–CN^- reagent has been developed. The highly fluorescent CBI derivative II was quantified by HPLC with fluorescence detection after separation on a non-silica-based polymeric column at elevated pH. The lowest limit of quantification was 5 ng/ml and required processing of 5 ml of urine. Similar assay methodology could be applied for the analysis of I in plasma.

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REFERENCES

1 N. C Rueppel, L A. Suba and J T. Marvel, *Biomed Mass Spectrom*, 3 (1976) 28
2 Z. Ismail, S. Aldous, E J. Triggs, B A Smithurst and H D Barry, *J Chromatogr*, 404 (1987) 372.
3 S E Meek and J Pietrzyk, *Anal. Chem*, 60 (1988) 1397
4 J D. DeMarco, S E Biffar, D G Reed and M A. Brooks, *J Pharmacol. Biomed. Anal*, 7 (1989) 1719
5 E Kwong, A M Y. Chiu, S McClintock and M Cotton, *Pharm Res*, 6 (Suppl) (1989) S-24, Abstract AP195
6 S. Bisaz, R Felix and H Fleisch, *Clin Chim Acta*, 65 (1975) 299
7 T. L Chester, E C Lewis, J J Benedict, R J Sunberg and W. C Tettenhorst, *J Chromatogr.*, 225 (1981) 17

8 G Flesch and S A Hauffe, *J Chromatogr*, 489 (1989) 446
9 P. T. Daley-Yates, L A Gillford and C R. Hoggarth, *J Chromatogr*, 490 (1989) 329
10 J P Fels, J Guyonnet, Y. Berger and W Cautreels, *J Chromatogr*, 430 (1988) 73
11 R G Carlson, K. Srinivasachar, R S Givens and B K Matuszewski, *J Org Chem.*, 51 (1986) 3978.
12 P DeMontigny, J F Stobaugh, R. S Givens, R G Carlson, K. Srinivasachar, L A Sternson and T Higuchi, *Anal Chem.*, 59 (1987) 1096.
13 B K Matuszewski, R. S. Givens, K Srinivasachar, R G. Carlson and T. Higuchi, *Anal Chem*, 59 (1987) 1102
14 P M. R DeMontigny, L. A Sternson, A J. Repta, J F. Stobaugh and T Higuchi, *U S Pat.*, 4 837 166 (1989), *C A*, 112 (18) (1990) 171496b.
15 C. A Parker, *Photoluminescence of Solutions*, Elsevier, New York, 1968, p 287
16 N Seiler, L Demish, K Blau and G S King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, London, 1977, Ch 9.
17 N. Seiler, *J. Chromatogr.*, 143 (1977) 221
18 J B F Lloyd, *J. Chromatogr.*, 178 (1979) 249
19 B. K. Matuszewski, R S. Givens, R. G. Carlson, T Higuchi, T. Kawasaki and O S. Wong, *U S Pat.*, 4 758 520 (1988), *C A*, 109 (11) (1988) 89347h.
20 R. S. Givens, R L. Schowen, J Stobaugh, T. Kuwana, F Alvarez, N Parekh, B K. Matuszewski, T Kawasaki, O. Wong, M. Orlovic, H Chokshi and K. Nakashima, in M. C Goldberg (Editor), *Luminescence Applications in Biological, Environmental and Hydrological Sciences*, American Chemical Society Symposium Series No 383, Washington, DC, 1989, Ch 8, pp 127–154.