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Liquid chromatographic determination of oestradiol in serum by pre-column derivatization with dansyl chloride or laryl chloride and peroxyoxalate chemiluminescence detection

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Abstract: Column liquid chromatography with chemiluminescence detection is used to determine oestradiol in serum. Oestradiol is labelled by means of a two-phase derivatization procedure with either dansyl chloride or laryl chloride. After derivatization the excess reagent is removed on a short amino-bonded column: the sulphonyl chloride functional group reacts with the amino groups causing removal of the label; the oestradiol derivatives are not retained. Chromatography of oestradiol derivatives is performed with methanol–water mixtures as eluent. Chemical excitation is carried out by adding bis(2-nitrophenyl)oxalate and hydrogen peroxide dissolved in acetonitrile to the column eluate. Linearity is observed ($r = 0.9998$; $n = 7$) over at least three decades (10^{-6}–10^{-9} M) for both dansyl chloride and laryl chloride. A detection limit of 5×10^{-10} M (50 fmol injected) has been achieved and the dansyl derivatization method has been applied to the determination of oestradiol in spiked serum.

Keywords: *Oestradiol; serum; derivatization; reversed-phase liquid chromatography; peroxyoxalate chemiluminescence.*

Introduction

The determination of oestrogens in biological fluids such as plasma and serum is important for the evaluation of placental and foetal functions in pregnant women [1]. The oestrogen levels vary from 0.1 ng ml^{-1} in the first trimester to 5–20 ng ml^{-1} in the third trimester of a pregnancy period. A dramatic fall in these levels can indicate foetal distress. In non-pregnant healthy women serum oestradiol concentrations have been reported to be in the sub-ppb range, i.e. as low as 0.05–0.01 ng ml^{-1} [2]. The determination of oestrogens in biological fluids was recently reviewed by Kabra [3]. In general, liquid–liquid or liquid–solid extraction procedures are used to collect the oestrogens from serum or plasma samples. Radioimmunoassay (RIA) methods are sensitive, but suffer from non-specificity and require either chromatography or solvent extraction prior to analysis [4]. As an alternative to RIA, analytical methods based on spectrophotometry, fluorimetry, gas chromatography and liquid chromatography (HPLC) have been developed [3, 5–8]. However, most of these methods lack either selectivity or sensitivity; even under optimal conditions the detection limits typically are 0.1–0.5 ng ml^{-1} or higher. Besides, several of the published procedures are rather time-consuming.

Recently, a fast and simple two-phase dansylation reaction for phenolic compounds has been developed by de Ruiter *et al.* [9]. Their labelling method was applied to the selective detection of the oestrogens, oestradiol and ethynyloestradiol [10]. The detection limits (4–11 ng), however, did not permit determination of the oestrogens at sub-ppb levels.

Peroxyoxalate chemiluminescence (CL) detection of dansylated compounds has often been shown to be more sensitive than fluorescence detection [11–13]. Nozaki *et al.* [14] described the CL detection of oestradiol in serum with detection limits of about 0.1 ng ml^{-1}. The derivatization procedure, however, requires 80 min at room temperature and for the detection system two additional pumps are required.

The aim of this study was to combine the fast

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derivatization procedure for oestradiol with a one-pump peroxyoxalate CL detection system. Furthermore, a comparison was made between dansyl chloride and Lissamine Rhodamine B sulphonyl (laryl) chloride. Laryl chloride is a promising label because of its excellent CL properties [15]. Besides, its emission wavelength of *ca* 590 nm allows the application of a 550-nm emission filter; this effects a distinct reduction in CL background [16]. The usefulness of a recently developed method for removal of excess dansyl chloride with an amino-bonded cartridge [17], is evaluated for laryl chloride.

Experimental

Chemicals

HPLC-grade solvents and C18-bonded SPE columns were purchased from Baker (Deventer, The Netherlands). Bis(2-nitrophenyl)oxalate (2-NPO) was synthesized as described in the literature [18]. 5-Dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride, see Fig. 1a) and tetrabutylammonium bromide (TBABr) were bought from Aldrich (Brussels, Belgium). Lissamine Rhodamine B sulphonylchloride (laryl chloride, see Fig. 1b) and tetrapentylammonium bromide were purchased from Kodak (Weesp, The Netherlands). Amino-bonded Bond Elut columns were purchased from Analytichem (Harbor City, CA, USA). Oestradiol (1,3,5[10]-oestratriene-3,17β-diol, see Fig. 1c) was obtained from Boehringer Mannheim (Mannheim, Germany). Stock solutions of oestradiol were prepared in methanol and kept in the dark at 4°C. All other chemicals were of analytical reagent grade.

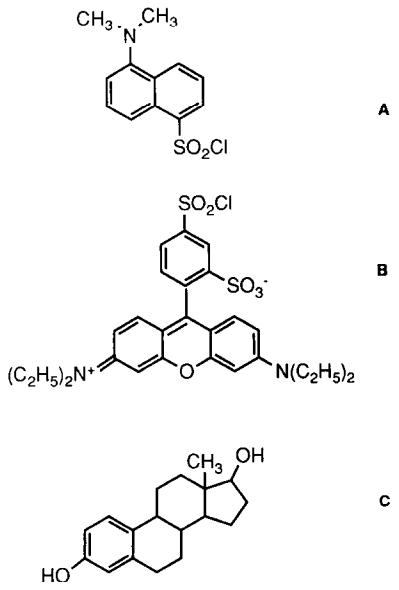

Figure 1
Structure of dansyl chloride (A), laryl chloride (B) and oestradiol (C).

Column liquid chromatography

The HPLC mobile phase flow of 0.5 ml min^{-1} was delivered by a Gilson (Villiers-le-Bel, France) gradient system consisting of a Gilson 305 pump (A), a Gilson 302 pump (B), a Gilson 805 manometric module and a 811B dynamic mixer. Pump A delivered methanol–100 mM imidazole buffer, pH 7.0 (97.5:2.5, v/v) and pump B methanol–2.5 mM imidazole buffer, pH 7.0 (2.5:97.5, v/v); both A and B contained 1 mM tetrapentylammonium bromide. For dansylated oestradiol the mobile phase contained 85% A and for larylated oestradiol 80% A. A Rheodyne six-port valve with a 100 μl loop was used for the introduction of samples on a 200 × 3.1 mm i.d. column packed with 3-μm LiChrosorb RP-18 (Merck, Darmstadt, Germany) by a slurry technique.

Detection system

Hydrogen peroxide and 2-NPO dissolved in acetonitrile at final concentrations of 50 and 5 mM, respectively, were mixed just before use and added to the column effluent with a pulseless Isco (Lincoln, NE, USA) μLC-500 syringe pump. The mobile phase (0.5 ml min^{-1}) and the reagent stream (0.3 ml min^{-1}) were mixed in a Valco T-piece just before the detection. An ATTO (Tokyo, Japan) AC 2220 CL detector (operated at 700 V) equipped with a 65 μl spiral flow-cell and a 470-nm cut-off filter for dansyl derivatives of a 550-nm filter for laryl derivatives was used for detection.

Derivatization of oestradiol with dansyl chloride or laryl chloride

To 500 μl of an aqueous oestradiol solution, adjusted to pH 12 with 1 M NaOH, 100 μl of an aqueous solution of tetrabutylammonium bromide (30 mg ml^{-1}, pH 12) and 600 μl of a solution of 0.1 mg ml^{-1} dansyl chloride in dichloromethane were added in a 100 × 9 mm reagent tube. The tube was capped with aluminium foil to avoid evaporation of dichloromethane and vortexed rigorously for 2 min. A 500 μl volume of the organic phase

were slowly introduced onto an amino-bonded Bond Elut column (previously washed with dichloromethane and dried with nitrogen or air) and the excess reagent, dansyl chloride, was allowed to react with the bonded amino groups for 10 min. The dansylated oestradiol was eluted from the Bond Elut column with 3 ml of dichloromethane; next, the organic eluate was gently evaporated to dryness and the residue dissolved in 500 μl of methanol–water (50:50, v/v). Finally, 100 μl were injected onto the C18 column.

With laryl chloride, the same procedure was followed as for dansyl chloride, except that desorption from the amino-bonded Bond Elut column was now carried out with 3 ml of dichloromethane–methanol (96:4, v/v), while the concentration of laryl chloride (in dichloromethane) was 0.1 mg ml^{-1} for high-level derivatizations (10^{-5}–10^{-7} M) and 0.02 mg ml^{-1} for low-level derivatization (10^{-7}–5×10^{-10} M).

Determination of oestradiol in spiked serum samples

Serum samples (1.0 ml) were mixed with 100 μl of 21% (w/v) phosphoric acid (final pH, *ca* 1.8). Spiking of the samples was performed by adding 100 μl of an aqueous oestradiol solution prior to the addition of phosphoric acid. Baker (No. 7020-01) 1 ml octadecylsilica disposable extraction columns were used for preconcentration and clean-up. After activating a column by 3 ml of methanol and flushing with 10 ml of water, the sample was preconcentrated and the cartridge flushed with 3 ml of water. Desorption was carried out with 600 μl of dichloromethane by applying some pressure. To the eluate, 100 μl of the dansyl chloride solution (1 mg ml^{-1}) in chloroform, 100 μl of the tetrabutylammonium bromide solution (30 mg ml^{-1}) in water (pH 12) and 500 μl of water (pH 12) were added and the derivatization procedure was carried out as described above.

Results and Discussion

Derivatization

The two-phase derivatization procedure for phenolic compounds using phase-transfer catalysis [9] was studied with dansyl chloride and laryl chloride as labels. For dansyl chloride, after derivatization the same conditions were used as before to remove excess reagent [17]: 500 μl of the organic layer are brought on a dry amino-bonded column where the excess dansyl chloride will react with the amino groups. The dansylated phenols are eluted from the column with dichloromethane, the eluate is evaporated to dryness, dissolved in mobile phase and injected on a C18 column.

The removal of excess laryl chloride on an amino-bonded cartridge is even more efficient than with dansyl chloride. The volume of organic phase that can be loaded onto the column is less critical than with dansyl chloride, because the retention of larylated oestradiol on amino-bonded silica is very high in pure dichloromethane or chloroform. After a 10-min reaction time, the amino column is washed with 1 ml dichloromethane–methanol (99:1, v/v) to remove relatively apolar side-products. Larylated oestradiol is desorbed with 3 ml dichloromethane–methanol (96:4, v/v). A subsequent washing of the column with pure methanol still does not elute the fluorescent red zone which is visible at the top of the column, indicating the presence of a reaction product with a stable sulphonamide bond. A single Bond Elut cartridge can be used at least 10 times, because only a small portion of the reactive amino groups is used up by the at most 50 μg of laryl chloride present after each derivatization.

Chromatography

For HPLC of dansylated oestradiol, methanol–water (85:15, v/v) containing imidazole buffer (pH 7.0) was used as eluent [17]. For larylated oestradiol, initially methanol–water (80:20, v/v) with the same imidazole buffer was applied. However, when derivatizing a blank and analysing at high sensitivity, large tailing peaks appeared in the chromatogram. These peaks are probably due to side-product formation during vortex mixing in dichloromethane in the presence of the strongly basic aqueous phase. A laryl chloride solution which was directly brought onto the amino column (i.e. without vortex mixing), eluted and analysed by HPLC, did not contain any of the interfering peaks. The tailing suggested the presence of amino groups in the side-products; therefore, 1 mM tetrapentylammonium bromide was added to the mobile phase in order to prevent interaction with silanol groups present in the packing material. Most of the tailing peaks now indeed shifted their position to the early part of the chromatogram, while the

retention of larylated oestradiol was not influenced.

Easily oxidizable compounds, such as iodide and bromide, have been reported to quench CL [19]; therefore, high concentrations of these ions cannot be tolerated. In the present work, 1 mM tetrapentylammonium bromide caused a small decrease in signal intensity but, fortunately, also in noise level; the S/N (signal-to-noise) ratio remained unchanged.

Detection system

Quenching of CL signal. Quenching of the luminescence of dansyl derivatives of phenolic compounds by electronegative (nitro or chloro) substituents, can be prevented by using a post-column photochemical reactor. After separation, the dansylated compounds are photochemically decomposed (in 5 s) and the reaction products, dansyl hydroxide and dansyl methoxide, are detected with high sensitivity [17, 20]. For a series of dialkylphenols, a six to nine-fold gain in sensitivity was obtained after irradiation of the dansylated phenols and fluorescence detection. However, CL detection of irradiated and non-irradiated dansylated oestradiol gave almost equal results. Neither did changing the irradiation time from 5 s to 10 or 20 s result in lower limits of detection. Obviously, the CL characteristics of the photoproducts are not superior to those of dansylated oestradiol.

For larylated phenolic compounds, post-column photolysis is not necessary either. In this case, the aromatic ring to which the sulphonyl chloride group is attached, serves as a spacer and prevents all influence on the luminescent ring system.

CL detection. The optimization of the CL detection system for dansylated phenolic compounds — resulting in the use of a 2.5 mM imidazole buffer (pH 7) in the methanol–water mobile phase, and the single-pump addition of the oxalate (2-NPO, 5 mM) and hydrogen peroxide (50 mM) in acetonitrile — was described in earlier papers [16, 17]. Using bis(2,4,6-trichlorophenyl)oxalate (TCPO), a relatively strong quenching effect has been noted in the presence of methanol [21]. In this study, using 2-NPO the S/N ratio in acetonitrile–water mixtures was three times higher than with methanol–water. Unfortunately, methanol–water mixtures had to be used to achieve a better separation of the dansylated oestradiol peak from the serum matrix. The stability of the oxalate 2-NPO in the presence of relatively low concentrations of hydrogen peroxide was satisfactory [22] and no precipitation problems occurred on addition of 2-NPO to the aqueous mobile phase, as is sometimes the case with TCPO.

For larylated oestradiol, the imidazole concentration in the methanol–water mobile phase was varied from 0.5 to 10 mM; 2.5 mM was found to be optimal. This result is slightly different from that reported for larylated chlorophenols in an acetonitrile–water mobile phase (10 mM imidazole [16]). This probably is a result of the faster CL kinetics in methanol compared to acetonitrile, making a lower catalyst concentration necessary [21, 23]. The influence of imidazole on the half-life of the CL reaction has been studied in detail by various authors [21, 24]: the larger the concentration of imidazole, the faster is the CL reaction.

Analytical data

The linearity of the complete system (derivatization, HPLC with CL detection) was checked for 10^{-6}–10^{-9} M oestradiol. For both labels, linearity was good ($n = 7$, $r = 0.9998$ for dansylated oestradiol and $n = 7$, $r = 0.996$ for larylated oestradiol). The repeatability found with a 5×10^{-7} M oestradiol solution was 3.5% (RSD, $n = 6$) for both labels.

To establish the limits of detection (LOD, based on signal-to-noise (S/N) ratio of 3), a 5×10^{-7} M oestradiol solution was derivatized with dansyl and laryl chloride, and was 100-fold diluted. For larylated oestradiol (detected using 550-nm emission cut-off filter), the LOD was five-fold better (1×10^{-10} M, 10 fmol injected) than for dansylated oestradiol (5×10^{-10} M, 50 fmol injected). The signal of larylated oestradiol was about three-fold higher and the noise about two-fold lower than for the dansylated derivative.

For low-level derivatization (500 μl of an aqueous oestradiol solution diluted before derivatization), however, the LOD was 5×10^{-10} M for both dansylated and larylated oestradiol. Using dansyl chloride, only a single small peak eluting just before the dansylated oestradiol peak appears in the chromatogram (see Fig. 2A). The LOD for larylated oestradiol is, however, negatively influenced by the appearance of several small peaks in the blank derivatization (cf. Fig. 2B). As a result, the

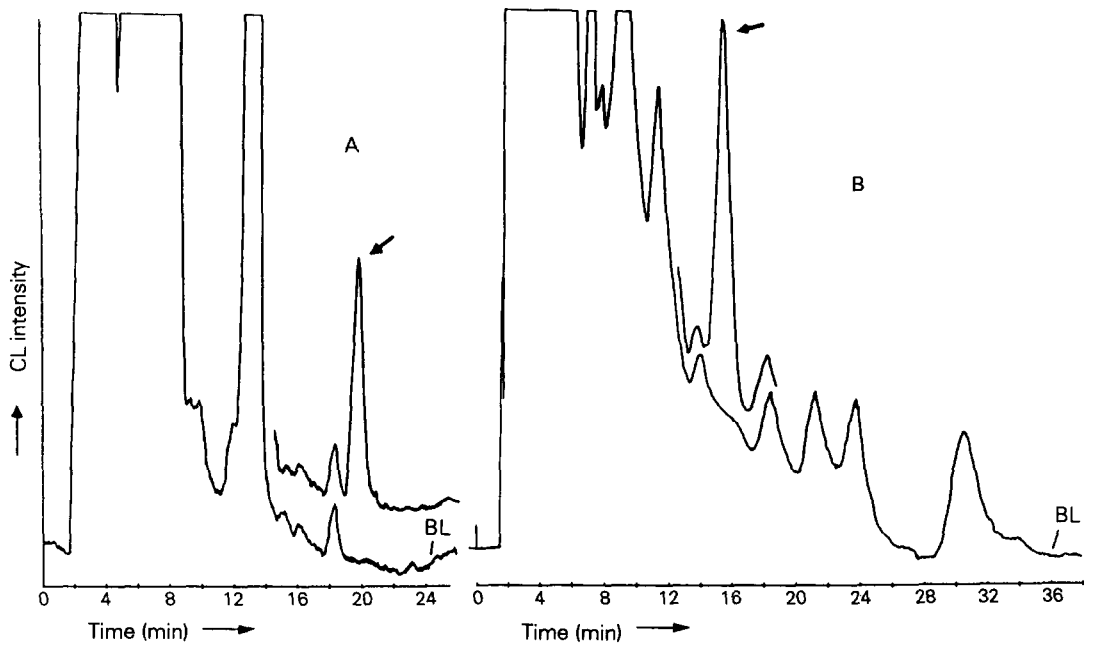

Figure 2
(A) HPLC with CL detection of a 500 μl standard solution of 10^{-8} M oestradiol (indicated by an arrow) and a blank solution (BL) derivatized with dansyl chloride. For derivatization, see Experimental. (B) HPLC with CL detection of a 500 μl standard solution of 10^{-8} M oestradiol (indicated by an arrow) and a blank solution (BL) derivatized with laryl chloride. For derivatization, see Experimental. Note that the sensitivity of the detector in (B) is two-fold lower than in (A).

determination of oestradiol in serum samples was carried out with dansyl chloride as label only.

Oestradiol in serum

In this study, 1.0 ml of serum was preconcentrated on a C18 cartridge and polar contaminants were washed off with 3 ml of water. Because the subsequent derivatization is performed in dichloromethane–water, oestradiol was eluted with 600 μl of dichloromethane. This eliminated a time-consuming evaporation step. The total procedure — i.e. preconcentration on the C18 cartridge, desorption, derivatization, evaporation to dryness and dissolution in methanol–water — only takes about 40 min.

The HPLC–CL chromatograms of a 1.0 ml serum sample spiked with 10^{-8} M oestradiol and of the corresponding blank are shown in Fig. 3. The oestradiol recovery was 80% in this case as well as with 5×10^{-8} M and 5×10^{-7} M oestradiol-spiked serum samples. Comparison of the chromatogram of Fig. 3 with that obtained for a spiked aqueous standard (Fig. 2A) clearly indicates that serum constituents do not cause any interference.

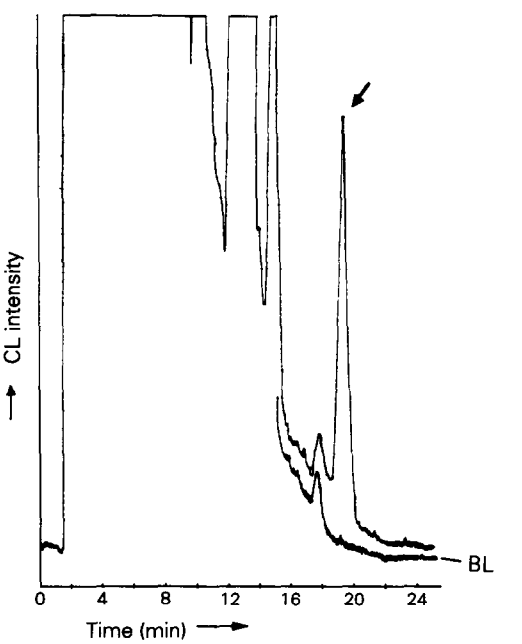

Figure 3
HPLC with CL detection of a 1.0 ml serum blank (BL) and 1.0 ml serum spiked with 10^{-8} M oestradiol (indicated by an arrow) and derivatized with dansyl chloride. For serum sample treatment, derivatization and further details, see Experimental.

Table 1
Characteristic data for HPLC analyses of oestrogens*

Steroid	Matrix	Sample pre-treatment	Detection mode	Detection limit ($ng\ ml^{-1}$)	Ref.
EN-gluc.	Standards	Sep-Pak extraction + deriv. (70°C, 30 min)	UV (328 nm)	10	7
ED, ET, EN	Urine	Hydrolysis (100 min) + two-step deriv. (35 min)	FL (350/415 nm)	0.4	8
ED, ET	Serum	Automated preconc.	FL (220/320 nm)	1	25
ET	Serum	Clin-Elut extraction	FL (280/308 nm)	1	26
ED, ET, EN	Urine	Hydrolysis + extraction	EC (+0.4 V)	1–10	27
ET	Serum	Ether extraction	EC (+0.75 V)	1	28
ED, EN	Serum	On-line preconc. (20 min)	EC (+1.0 V)	0.3	29
ED	Serum	Bond Elut extraction + deriv. (total 130 min)	PO-CL	0.1	14
ED	Serum	Bond Elut extraction + deriv. (total 40 min)	PO-CL	0.06	This work

*Abbreviations used: ED, oestradiol; ET, oestriol; EN, oestrone; gluc., glucuronide; deriv., derivatization; FL, fluorescence detection; EC, electrochemical detection; PO-CL, peroxyoxalate chemiluminescence detection.

This demonstrates the high selectivity of the present method. Not surprisingly, the LODs of the standards and serum samples are essentially the same. Actually, the LOD is lower in the latter case (3×10^{-10} M), because 1.0 ml serum was processed as against 500 µl of the standard. Characteristic data from a number of recent studies on the HPLC determination of oestrogens (oestradiol, oestriol and oestrone) in biological fluids are summarized in Table 1. It is obvious that all methods using UV absorbance, native fluorescence or electrochemical detection lack sensitivity, and that derivatization techniques are necessary to effect a substantial decrease of the detection limits. The method described in this paper offers a further improvement in this respect (although an additional three to five-fold increase in sensitivity is certainly desirable); besides it is rather fast. A comparison with gas chromatography–mass spectrometry (GC–MS) methods for oestrogens [6] or a structurally similar hormone [30] in serum, shows that the clean-up procedure in the present study is considerably faster and that the detection limit is as good as with GC–MS.

Conclusions

The practicability of a fast two-phase derivatization procedure for the trace-level determination of oestradiol in serum followed by HPLC with peroxyoxalate CL detection is demonstrated. With dansyl chloride as label, 3×10^{-10} M (0.06 ng ml^{-1}; 50 fmol injected) oestradiol can be detected in serum. In principle, this allows HPLC monitoring of oestradiol levels during the total pregnancy period. Compared with other procedures reported in the literature, the present method does not suffer from specificity problems as does RIA, and it is at least 10 times more sensitive than methods using electrochemical or (native) fluorescence detection. The method described by Nozaki *et al.* [14] yields a comparable detection limit, but it requires a rather lengthy clean-up and derivatization procedure, and the set-up used is rather expensive, because two additional reagent pumps have to be used.

Although laryl chloride allows, in principle, five-fold more sensitive detection of oestradiol than does dansyl chloride, reagent impurities and/or side-products formed during the two-phase reaction (even in standard solutions), mar the result. As a result, the detection limit obtained with laryl chloride is at least as high as that for dansyl chloride.

The removal of excess reagent with an amino-bonded cartridge works well for both dansyl and laryl chloride. This approach will be extended to other labelling procedures in the near future.

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References

[1] U. Goebelsmann, R.K. Freeman and J.M. Westman, *Am. J. Obstet. Gynaecol.* **115**, 795–798 (1973).

[2] D. Both, in *Analytical Profiles of Drug Substances* (K. Forey, Ed.), Vol. 12, Ch. 5, pp. 140–142. Academic Press, New York (1983).
[3] P.M. Kabra, *J. Chromatogr.* **429**, 155–176 (1988).
[4] M. Pazzagli and M. Serio, in *Handbook of Experimental Pharmacology* (C. Patrono and B.A. Peskar, Eds), Vol. 82, *Radioimmunoassay in Basic and Clinical Pharmacology*, p. 390. Springer, Berlin (1987).
[5] L.A. Kaplan, in *Methods in Clinical Chemistry* (A.J. Pesce and L.A. Kaplan, Ed.), pp. 1043–1044. C.V. Mosby, St Louis, MO, USA (1987).
[6] K.-Y. Tserng, R.K. Danish and J.S. Bendt, *J. Chromatogr.* **272**, 233–241 (1983).
[7] S. Chakir, P. Leroy, A. Nicolas, J.-M. Ziegler and P. Labory, *J. Chromatogr.* **395**, 553–561 (1987).
[8] J. Ishida, M. Kai and Y. Ohkura, *J. Chromatogr.* **431**, 249–257 (1988).
[9] C. de Ruiter, R.R. Otten, U.A.Th. Brinkman and R.W. Frei, *J. Chromatogr.* **436**, 429–436 (1988).
[10] C. de Ruiter, J.N.L. Tai Tin Tsoi, U.A.Th. Brinkman and R.W. Frei, *Chromatographia* **26**, 267–273 (1988).
[11] S. Kobayashi and K. Imai, *Anal. Chem.* **52**, 424–427 (1980).
[12] K. Imai and R. Weinberger, *Trends in Anal. Chem.* **4**, 170–175 (1985).
[13] G.J. de Jong and P.J.M. Kwakman, *J. Chromatogr.* **492**, 319–343 (1989).
[14] O. Nozaki, Y. Ohba and K. Imai, *Anal. Chim. Acta* **205**, 255–260 (1988).
[15] K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta* **177**, 111–120 (1985).
[16] P.J.M. Kwakman, J.G.J. Mol, D.A. Kamminga, R.W. Frei, U.A.Th. Brinkman and G.J. de Jong, *J. Chromatogr.* **459**, 139–149 (1988).
[17] P.J.M. Kwakman, D.A. Kamminga, U.A.Th. Brinkman and G.J. de Jong, *J. Chromatogr.* **553**, 345–356 (1991).
[18] A.G. Mohan and N.J. Turro, *J. Chem. Educ.* **51**, 528–529 (1974).
[19] C. Gooyer and N.H. Velthorst, *Biomed. Chromatogr.* **4**, 92–95 (1990).
[20] C. de Ruiter, J.F. Bohle, G.J. de Jong, U.A.Th. Brinkman and R.W. Frei, *Anal. Chem.* **60**, 666–670 (1988).
[21] N. Hanaoka, R.S. Givens, R.L. Schowen and T. Kuwana, *Anal. Chem.* **60**, 2193–2197 (1988).
[22] K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta* **177**, 103–110 (1985).
[23] R. Weinberger, *J. Chromatogr.* **314**, 155–165 (1984).
[24] M. Orlovic, R.L. Schowen, R.S. Givens, F. Alvarez, B. Matuszewski and N. Parekh, *J. Org. Chem.* **54**, 3606–3610 (1989).
[25] T. Dohji, M. Fuchimi, T. Kawabe, F. Kamiyama, M. Mori, N. Sugita and O. Tanizawa, *J. Chromatogr.* **311**, 249–255 (1984).
[26] P.M. Kabra, F.W. Tsai and L.J. Martin, *Clin. Chim. Acta* **128**, 9–14 (1983).
[27] H. Gunasingham, B.T. Tay and K.P. Ang, *J. Chromatogr.* **341**, 271–278 (1985).
[28] L.A. Kaplan and D.C. Hohnadel, *Clin. Chem.* **29**, 1463–1468 (1983).
[29] Y. Suzuki, N. Hayashi and K. Sekiba, *J. Chromatogr.* **426**, 33–40 (1988).
[30] T. Järvinen, T. Keinonen, S. Auriola and P. Peura, *J. Chromatogr.* **495**, 13–20 (1989).

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