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Evaluation of amperometric detection for the liquid-chromatographic determination of tetracycline antibiotics and their common contaminants in pharmaceutical formulations

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Abstract

A sensitive high-performance liquid chromatographic technique with amperometric detection has been developed for the determination of seven commercially used tetracyclines in bulk powders and pharmaceutical preparations. The technique is based on the oxidation of these compounds and their contaminants at the glassy carbon electrode. The extraction procedures are simple and the HPLC conditions separate the tetracyclines from their major degradation products. The chromatography was performed using a commercially available octadecylsilane column, with a mobile phase: KH_2 PO_4 (pH = 2.5; 0.05 M) – acetonitrile (84:16, v/v) and detection at 1.2 V. The technique permits the simultaneous determination of trace amounts of chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, oxytetracycline and tetracycline as well as the separation of their common impurities (epi, anhydro and epianhydro contaminants) with detection limits of 0.1–1.0 ng μl^{-1} and recoveries of 99.1–100.4%. No interference was observed from the commonly present excipients in pharmaceutical formulations. © 1997 Elsevier Science B.V.

Keywords: Tetracyclines; Pharmaceutical formulations; Reversed-phase high-performance liquid chromatography; Amperometric detection; Tetracycline degradation products

1. Introduction

The tetracycline antibiotics are active against a wide range of Gram-positive and Gram-negative bacteria, being widely used in human and veterinary medicines as well as feed additives. Nine tetracyclines are commercially available, of which chlortetracycline (CTC), demeclocycline (DMC), doxycycline (DC), methacycline (MTC), minocycline (MNC), oxytetracycline (OTC), rolitetracycline and tetracycline (TC) are permitted for human administration.

Many methods have been described for the determination of tetracyclines in pharmaceutical preparations and various biological matrices. While the official methods of assay retain the microbiological approach [1,2], these methods are not only expensive and time consuming but also are poor in terms of sensitivity and specificity. A

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further problem of the bioassay is that the degradation products or contaminants of tetracyclines, such as epi-, anhydro- and epianhydro-tetracyclines, which are present in varying amounts in the raw materials and finished products of tetracyclines, may also have antimicrobial properties. Thus the results of bioassay will not necessarily be a true representation of the antimicrobial potency of the labelled material [3]. Under abnormal conditions (heat, pH, humidity), tetracyclines undergo reversible epimerisation at C-4 to form a mixture of epi-, anhydro-, epianhydro and tetracyclines. The epimer has antibiotic activity only 2–5% of that of the parent tetracycline [4]. These degradation products, specially the epianhydrotetracyclines, shown definite toxicity to renal tubular function and initiate a reversible Fanconi-Type syndrome, characterised by polyuria, polydispepsia, vomiting, proteinuria, aminoaciduria and acidosis [5]. From this viewpoint and owing to the toxicity and probable inactivity of the impurities and degradation products, it is very important that their contents can be controlled simply and precisely.

There has recently been a move to replace the biological assay by other methods, in particular, high performance liquid chromatography (HPLC) in order to achieve the sensitivity and specificity to detect tetracyclines at low concentration levels [6–12]. Croubles and Van Peteghen used a spectrofluorimetric measurement by a post-column complexation of tetracyclines with zirconium ion [6]. McCracken et al. reported recently a sensitive liquid chromatographic method with fluorescence detection after a post-column derivatisation with aluminium ion [7]. Despite the usefulness of these methods, they require special attention and may be time consuming in their application to practical problems in pharmaceutical analysis. A number of other assay procedures based on HPLC, in particular with spectrophotometric detection, have appeared in the literature [8–12]. Except for one report concerning the electrochemical behaviour of four tetracyclines [13], these drugs have not been investigated by HPLC using amperometric detection. Although electrochemical detection in HPLC is generally regarded as being more technically demanding than UV detection, it provides a much higher degree of selectivity compared with UV or fluorescence detection, it usually requires less sample and is therefore often recommended as an alternative method for sensitive detection.

This paper describes a rapid HPLC method with amperometric detection for the separation and determination of tetracycline antibiotics and their major impurities, a method designed to be suitable for the quality assessment of these drugs in pharmaceutical formulations.

2. Experimental

2.1. Materials

Reference standards for tetracycline-HCl, oxytetracycline-HCl, doxycycline-HCl, minocycline-HCl, chlortetracycline-HCl, methacycline-HCl and demeclocycline-HCl were obtained from the United States Pharmacopoeial Convention (Rockville, MD, USA). The degradation product 4-epitetracycline was a gift from Wyeth-Lederle (Sydney, Australia) and anhydro- and 4-epianhydro-tetracycline were obtained from Janssen Chimica (Beerse, Belgium). Bulk materials for tetracyclines were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Pfizer (Sydney, Australia), Cyanamid (Sydney, Australia), Hakim (Tehran, Iran) and Razak (Tehran, Iran). Dosage forms were obtained from local pharmacies as follows: chlortetracycline-HCl (Lederle Aureomycin 3% ointment), demeclocycline-HCl (Lederle 300 mg Declomycin tablets), doxycycline-HCl (Alphapharm 100 mg Doxylin-100 tablets and Razak 100 mg Doxycycline-hyclat tablets), methacycline-HCl (Warner Rondomycin 300 mg capsules), Minocycline-HCl (Lederle 100 mg Minomycin capsules), oxytetracycline-HCl (Razak 250 mg Oxytetracycline capsules) and tetracycline-HCl (Lederle 250 mg Achromycin capsules, Hakim 250 and 500 mg Tetracycline capsules and Razak Tetracycline topical and ophtalmic ointments).

The acetonitrile used was ChromAR HPLC grade (Mallinckrodt). Potassium dihydrogen orthophosphate (BDH) and orthophosphoric acid

(BDH) were analytical reagent grade. Water was double-distilled from an all-glass apparatus.

2.2. Chromatography

Analyses were carried out with a Perkin-Elmer Series 4 solvent delivery system (Perkin-Elmer, Bodensee, Germany), equipped with an Rheodyne Model 7125 injector and a 5-μl loop (Rheodyne, Cotati, CA, USA), a BAS Model 4 or 4A electrochemical detector with a glassy carbon working electrode (Bioanalytical System, West Lafayete, IN, USA) operated at 1.2 V with respect to an Ag/AgCl reference electrode. Detector signals were processed with a Perkin-Elmer LC-100 integrator. The analytical separation was achieved on a 300×4.6-mm octadecylsilane column, 10 μm particle size (μ-Bondapak C_{18}, Waters Associates, Milford, MA, USA) with the mobile phase: potassium dihydrogen phosphate buffer (pH 2.5; 0.05 M) – acetonitrile (84:16, v/v) at a flow-rate of 1.0 ml min^{-1}.

2.3. Preparation of standards and samples

The tetracycline stock solutions were prepared from reference standards and samples of bulk drug by accurately weighing about 10 mg substance and dissolving in 100 ml of mobile phase in a volumetric flask. When stored at 4°C, these stock solutions were stable for approximately two days. The working standards were prepared from the stock solutions by dilution with the appropriate volume of mobile phase to make the final concentrations in the range 0.1–50 ng μl^{-1}.

Tablets were prepared for analysis by crushing 20 tablets with a mortar and pestle. For capsule dose forms, the contents of 20 capsules were pooled. An accurately weighed portion of the powder, equivalent to the antibiotic content of one tablet or capsule, was transferred to a 100 ml volumetric flask. After adding 50 ml of mobile phase, the solution was sonicated for 10 min to aid dissolution and diluted with mobile phase to volume. A portion of this solution was filtered through a 0.45-μm nylon filter and an aliquot of filtrate was diluted with the appropriate volume of mobile phase to make a final concentration in the range of 5–10 ng μl^{-1}).

Ointments were prepared for analysis by transferring an accurately weighed portion of ointment equivalent to 1.0 mg tetracycline into a separatory funnel. The ointment was dissolved in 5 ml cyclohexane or ether and the drug extracted from the organic layer with 3×5 ml portions of mobile phase. The aqueous layers were combined in a 100 ml volumetric flask and brought to volume with mobile phase.

3. Results and discussion

Each tetracycline contains a phenolic substituent on position 10 and a dimethylamino substituent on position four. In amperometric detection, the oxidation of the tetracyclines occurs through these moieties. The optimum working potential for the amperometric detection was chosen at 1200 mV. This potential, which assures the oxidation of the tetracyclines, was determined from the so-called hydrodynamic voltammograms (Fig. 1). The voltammograms were obtained by injecting fixed volumes of the standard solution

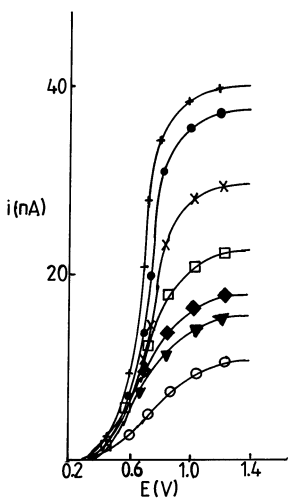

Fig. 1. Hydrodynamic voltammograms of the tetracyclines injected at a concentration of 10 ng μl^{-1} (+) MNC; (●) OTC; (X) TC; (□) DMC; (◆) MTC; (▼) DC and (○) CTC. Chromatographic conditions: column 10 μm μ-Bondapak C_{18} (300×4.5 mm I.D); mobile phase, potassium dihydrogenphosphate buffer (pH 2.5, 0.05 M) – acetonitrile (84:16, v/v); flow-rate, 1.0 ml min^{-1} and injection volume 20 μl.

and varying the potential between 600 and 1300 mV. Owing to the high background current, operation at higher potential would be disadvantageous for quantification of these compounds [13].

Electrochemical detection necessitates a mobile phase with a buffer in the low ionic strength range to provide adequate conductivity while minimising the contribution to background current. Phosphate buffer offered the best sensitivity in regards to the studied compounds, compared with other buffers tested.

Due to their highly polar nature, tetracyclines are normally determined by reversed-phase HPLC using either a silica based or polymeric ODS column [14–17]. The use of polymeric columns has been mostly in conjunction with an elevated column temperature, and required the use of viscous organic modifiers, and alkaline mobile phases containing additives such as EDTA and/or tetrabutylammonium ion pairing agents [18–22]. In this paper the separation was performed using a commercially available ODS column, with no disadvantage compared to the previously recommended polymeric one.

The pH of the mobile phase was chosen as 2.5 to minimise the formation of isomeric analogues, which occurs rapidly in alkaline medium [12]. The final concentration of acetonitrile chosen for the mobile phase was 16%, which allowed the tetracyclines to elute with favourable retention times while maintaining adequate resolution between the parent compounds and their major contaminants.

Typical chromatographic separation of tetracyclines and major contaminants are shown in Figs. 2 and 3. From these chromatograms, it can be concluded that the technique used here would be useful for the general screening or identification of tetracycline samples. The retention times of the tetracyclines as listed in Table 1 were relatively short with a maximum run time of 30 min being required in the case of doxycycline. Under the conditions described, a better separation of the studied tetracyclines from each other and from their common contaminants was achieved, compared to that of the USP XXIII method. Improved peak symmetry and reduced base widths of peaks are also features of the present method,

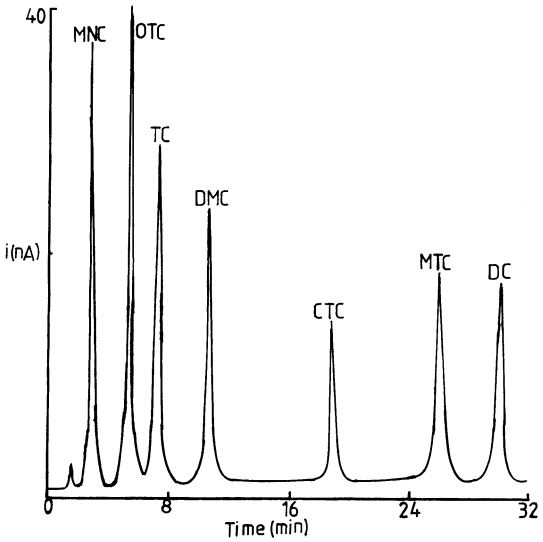

Fig. 2. Separation of various tetracyclines using HPLC-ECD. Chromatographic conditions are as described in Fig. 1.

along with the improved resolution of the impurities. Comparison of capacity ratio and theoretical plate values with the British Pharmacopoeia method indicate that the reversed phase column gives superior resolution to the ion-exchange column employed in the British Pharmacopoeia for the HPLC-assay of tetracyclines. The electrochemical method is capable of detecting the common contaminants in tetracyclines at levels lower

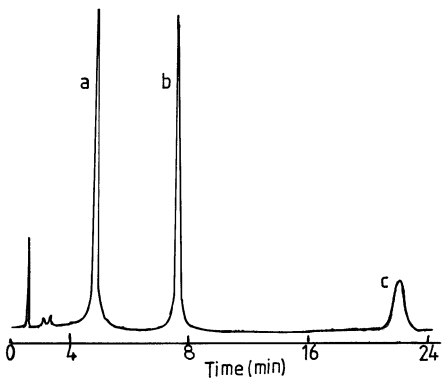

Fig. 3. Representative chromatogram of tetracycline degradation products: (a) epi-tetracycline, (b) epi-anhydrotetracycline, and (c) anhydrotetracycline. Chromatographic conditions are as described in the text.

Table 1
Retention times in minutes for HPLC-ECD analysis of tetracyclines and their contaminants

Antibiotics		Contaminant		
		Epi	Epianhydro	Anhydro
MIN	2.70	–	–	–
OTC	5.40	3.6	9.5	20.8
TC	7.40	5.5	11.0	22.2
DMC	10.85	–	–	–
CTC	18.70	16.2	22.0	34.5
MTC	26.00	–	–	–
DC	29.80	–	–	–

Chromatographic conditions are given in the text.

than that specified in the current official USP and BP methods [1,2]. The main advantage of the method is that the results can be obtained rapidly. Replicates of samples and standards can be run in a few hours and the degree of contamination can be calculated immediately. On the other hand, in the biological assay, a degraded sample when compared to the standard will give a potency ratio less than 1, which will make the assay method statistically unacceptable.

3.1. Assay validation

A series of validation tests were performed on this HPLC method. The calibration curves were constructed separately for each drug. The correlation coefficients, slope and y-axis intercept were calculated according to an equation of the form ($y = m\,x + b$) which was then used to calculate the concentrations in the samples. The calibration graphs of peak-height (in mm) versus concentration (in ng μl^{-1}) showed linear relationship between 0.1 and 50 ng μl^{-1} for DC, MNC, OTC and TC and relationship between 0.5–50 ng μl^{-1} for CTC, DMC and MTC. Least-squares linear regression analysis gave the values for slopes, intercepts and correlation coefficients shown in Table 2.

3.1.1. Precision

The intra- and inter-day precision of the method was determined for both the retention times and peak heights by repeated analysis (seven identical injections) of tetracycline standard solutions on each of 4 days. The intra-day relative standard deviation (RSD) values for retention times were 0–0.62% and for peak heights 0.3–1.4%. To assess the inter-day precision of the method, fresh standards were prepared each day since solutions of the tetracyclines are not sufficiently stable to allow use over a number of days. The results obtained for each day were normalised to the concentration used on the first day and from these the RSD values for peak height and retention times were 1.1–2.4% and 0.2–0.7% respectively. The reproducibility of the assay was evident also from this test.

3.1.2. Specificity

The specificity of the proposed method for determination of chlortetracycline and tetracycline in the presence of the major degradation products like epi-, anhydro and epi-anhydrotetracycline was assessed by spiking the tetracycline samples with these impurities. There was adequate resolution of all these compounds. The specificity was further determined by analysis of standard solutions of CTC and TC, which had been stored at 70°C for 10 h. Fig. 4 shows the presence of decomposition peaks along with undecomposed tetracycline. The peaks from the decomposition products were individually identified. Thus the proposed method is capable of resolving tetracyclines from the degradation products generated during manufacture or storage.

Table 2
Calibration curve parameters for HPLC-ECD analysis of tetracyclines obtained by linear regression analysis of peak height in mm vs. concentration in ng μl^{-1}

Tetracyclines	Slope	Intercept	Correlation coefficient
CTC	0.361	0.334	0.9996
DC	0.369	0.605	0.9997
DMC	0.457	0.902	0.9997
MNC	1.009	0.028	0.9999
MTC	0.498	0.943	0.9998
OTC	1.067	0.170	0.9999
TC	1.079	0.192	0.9999

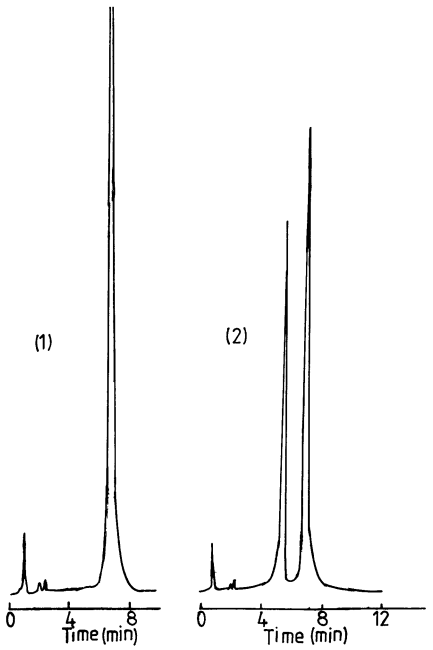

Fig. 4. A typical chromatogram from the HPLC-ECD analysis of tetracycline HCl bulk drug substance in aqueous solution: (1) immediately after preparation of the solution; (2) after storage of the solution at 70°C for 20 min.

3.1.3. Recovery, reproducibility and limit of detection

The recovery of the HPLC-ECD method was checked by analysing both aqueous solutions and placebo samples spiked with known amounts (25% of the claimed contents) of each of the drugs studied here. Essentially quantitative recoveries were obtained in each instances. The reproducibility was determined by consecutively injecting seven aliquots of standard solutions for each tetracycline as mentioned above. The limit of detection of the assay has been calculated to be 0.1–1 ng μl^{-1}, yielding a detector response approximately equal to three times the detector noise.

Validation of the HPLC-ECD method was completed by assaying several commercially available tetracycline formulations from different manufacturers. Analysis by this HPLC technique demonstrated the content of active compounds to correspond to the label, but revealed the existence of impurity in the form of the degradation products mentioned above. The assay was carried out six times for each type of preparation with different samples taken for each assay. The results of these assays are shown in Table 3. The quantification of CTC was performed only by the HPLC-ECD method. With respect to the content of impurities and degradation products, the assays did not confirm in all cases that the formulations are of compendial quality. Quantitative measurements of the potential contaminants and degradation products like ETC, ATC and EATC were performed on tetracyclines bulk drug substances and formulations. The bulk samples contained small amounts of impurities, which were below the limit ranges established by USP and BP (2–3% EATC) The greatest number and percentage of these impurities and degradation products were found in OTC and TC formulations, especially those not manufactured in Australia. In one cases of OTC capsules, which were still within their nominated expiry date, we found the significant amount of 50% C 4 epimer (Fig. 5).

The general advantage of this method is that all the tetracyclines described herein can be separated and determined in a single run. The method differentiates the tetracyclines from their contaminants and degradation products, so the quantification of epi-, anhydro- and epianhydro contaminants was achieved under the conditions described.

In conclusion, the proposed HPLC-ECD method is precise, reproducible and specific for individual tetracyclines and can be applied for purity, identification, quantification and, where required, the dissolution behaviour of tetracyclines in pharmaceutical formulations which could be adapted by quality control laboratories. In addition the method is suitable for the screening of formulated samples, which contain other pharmacologically active, but electrochemically inactive ingredients (e.g., for the composition of tetracycline-HCl with novobiocin-sodium and prednisolone or demeclocycline-HCl with nystatin). The simplification of solvent conditions and the use of a commercially available reverse-phase column with the sensitive electrochemical detector are advantages which make the method reported here a suitable alternative to biological

Table 3
Analysis of tetracycline-HCl dosage forms by the proposed HPLC-ECD and pharmacopoeial methods

Formulation		Label amount	% Found, ± SD ($n = 6$)	
			HPLC-ECD method	Official method
DC	Doxylin tablet	100 mg	97.8 ± 2.7	98.0 ± 2.7[a]
	Doxycycline hyclate tablet	100 mg	98.3 ± 1.9	98.6 ± 2.4b
DMC	Declomycin tablet	300 mg	100.2 ± 2.0	98.2 ± 2.9[b]
MTC	Rondomycin capsule	300 mg	99.0 ± 1.9	98.7 ± 2.0[b]
MNC	Minomycin capsule	100 mg	103.7 ± 1.9	105.0 ± 2.9[b]
OTC	Oxytetracycline capsule	250 mg	52.0 ± 1.6	49.9 ± 2.1[b]
TC	Achromycin tablet	250 mg	99.1 ± 1.4	98.2 ± 2.1[a]
	Tetracycline-HCl tbl.	250 mg	102.1 ± 1.4	105.1 ± 1.9[b]
	Tetracycline-HCl tablet	500 mg	106.5 ± 1.7	107.9 ± 2.2[a]
CTC	Aureomycin ointment	3%	98.5 ± 2.2	Microbial assay[a,b] (not performed)

[a]BP 1993.
[b]USP XXIII.

assay for both bulk and dosage forms of all tetracyclines.

Preliminary studies carried out on spiked plasma samples extracted by a solid-liquid procedure using Sep-Pak C_{18} cartridges, indicate the applicability of this method for biological samples and pharmacokinetic studies. The similarity in electrochemical and chemical properties of the compounds allows the selection of one tetracycline to be the internal standard for the assay of others.

The HPLC-ECD assay method was also tested for use in dissolution studies. Distilled water (900 ml) at 37 ± 0.5°C was used with the USP rotating paddle at 75 rpm. A 5 ml aliquot of the medium was withdrawn after the time required in monograph for each individual tetracycline- HCl in capsule or tablet form (30–60 min) and filtered through a 0.45 μm membrane filter. One ml portions of the filtrate were diluted with the mobile phase to make a 10-ng μl^{-1} working solution of each tetracycline. A 5-μl portion of the working solution was injected into the liquid chromatograph under the conditions specified above for the assay. Similarly 5 μl of the tetracycline standard solution were injected and the amount of dissolved tetracycline was quantified by comparison of the peak heights obtained for the standard and sample chromatograms.

Results for the studied tablets and capsules varied from 81 to 94% dissolved of the labelled amount of the individual tetracyclines, depending on the particular active compound and manufacturer. In one case (Hakims Tetracycline Tablets) the dissolution rate was significantly lower than the pharmacopoeial requirement (28% of the labelled amount released in 60 min).

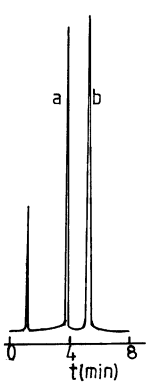

Fig. 5. Chromatogram from the HPLC-ECD analysis of a sample of oxytetracycline capsules: (a) epi-oxytetracycline and (b) oxytetracycline.

References

[1] United States Pharmacopoeia XXIII, United States Pharmacopoeial Convention, Rockville, MD, USA, 1995.

[2] British Pharmacopoeia, HMSO, London, 1993.
[3] A. Ray, R. Harris, J. Chromatogr. 467 (1989) 430–435.
[4] L.A. Mitscher, The Chemistry of the Tetracycline Antibiotics, Medical Research Series, Vol. 9. Marcel Dekker, New York, 1978.
[5] J.M. Gross, Ann. Int. Med. 58 (1963) 523–528.
[6] W. Croubles, C.V. Petheghem, W. Baeyens, Analyst. 119 (1994) 2713–2716.
[7] R.J. McCracken, W.J. Blanchflower, S.A. Haggan, D.G. Kennedy, Analyst. 120 (1995) 1763–1766.
[8] D.M. George, B.A. Raymond, J. Chromatogr. Sci. 16 (1978) 93.
[9] J.Y.C. Hon, L.R. Murray, J. Liq. Chromatogr. 5 (1982) 1973.
[10] W.N. Barnes, A. Ray, L.J. Bates, J. Chromatogr. 347 (1985) 173.
[11] Y. Vander Heyden, K. Luypaert, C. Hartmann, D.L. Massart, J. Hoogmartens, J. De Beer, Analytica. Chimica. Acta. 312 (1995) 245–262.
[12] P.D. Bryan, J.T. Stewart, J. Pharm. Biomed. Anal. 11 (1993) 971–976.
[13] W. Hou, E. Wang, Analyst 114 (1989) 699–702.
[14] N.H. Khan, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Pharm. Biomed. Anal. 7 (1989) 339–353.
[15] K. Dihuidi, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr. 246 (1982) 350–355.
[16] J.H. Knox, J. Jurand, J. Chromatogr. 186 (1979) 763–782.
[17] M. Skolic, B. Filipovic, M. Pokorny, J. Chromatogr. 509 (1990) 189–193.
[18] N.H. Khan, P. Wera, E. Roets, H. Vanderhaeghe, J. Chromatogr. 385 (1986) 444–447.
[19] W. Naidong, E. Roets, J. Hoogmartens, J. Pharm. Biol. Anal. 7 (1989) 1691–1703.
[20] N.H. Khan, P. Wera, E. Roets, J. Hoogmartens, J. Liq. Chromatogr. 13 (1990) 1351–1374.
[21] W. Naidong, K. Verresen, E. Roets, J. Hoogmartens, J. Chromatogr. 586 (1991) 61–66.
[22] J.W. Muritu, I.O. Kibwage, C.K. Maitai, J. Hoogmartens, J. Pharm. Biomed. Anal. 12 (1994) 1483–1488.

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