Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, Nos 10–12, pp. 845–850, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Liquid chromatography for the quantitative analysis of antibiotics — some applications using poly(styrene-divinylbenzene)*

J. HOOGMARTENS

Katholieke Universiteit Leuven, Instituut voor Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie, Van Evenstraat 4, B-3000 Leuven, Belgium

Abstract: Problems arising from the microbiological assay of antibiotics are discussed. The existence of several systems to express the potency leads to confusion. The use of potency to express the content of bulk products can lead to difficulties in the interpretation of the content of pharmaceutical preparations. Such problems can be avoided if the content of antibiotics is expressed as percentage weight in weight. This involves the application of selective assay methods such as liquid chromatography. The reproducibility of liquid chromatography depends largely on the quality of the stationary phase. Use of poly(styrene-divinylbenzene) as the stationary phase can lead to good reproducibility as is reported for erythromycin and for the tetracyclines, of which minocycline is discussed as an example.

Keywords: *Antibiotics; microbiological assay; liquid chromatography; poly(styrene-divinylbenzene); erythromycin; minocycline.*

Systems Used to Express the Potency of Antibiotics

The microbiological assay of antibiotics and its replacement by a liquid chromatographic (LC) method has been discussed by other workers [1, 2]. The total potency of a sample as determined by microbiological assay is most often expressed in International Units per milligram (IU mg^{-1}). This international system is used by the World Health Organization and by most pharmacopoeias, e.g. the European Pharmacopoeia (Ph. Eur.) [3]. In the USA another system is used by the Federal Drug Administration and the United States Pharmacopeia (USP) [4]. There the potency is usually expressed in micrograms per milligram (μg mg^{-1}); for some antibiotics, however, the potency is expressed in Units per milligram (U mg^{-1}). It should be emphasized that the term 'micrograms' in this expression represents micrograms of activity and cannot be interpreted as micrograms of weight. No relation exists between the International and the American systems and each system uses its own reference substance. This implies that an American reference substance expressed in μg mg^{-1} cannot be used to express the potency in IU mg^{-1}, nor can it be used to express a content in % w/w. In Europe only the Ph. Eur. or WHO reference substances expressed in IU mg^{-1} can be used.

It should also be emphasized that units or micrograms have no absolute value. Each reference substance therefore leads to a particular system of units; no equivalence exists between 1 IU of antibiotic A and 1 IU of antibiotic B. This can be illustrated by the following examples from the Ph. Eur. and the USP.

For polymyxin B sulphate (mainly polymyxin B) a minimal potency of 6500 IU mg^{-1} is required by the Ph. Eur. and 6000 U mg^{-1} by the USP. For the closely related colistin sulphate (mainly polymyxin E) the minimal potency prescribed by the Ph. Eur. is 19000 IU mg^{-1} and 500 μg mg^{-1} by the USP. This does not mean that the Ph. Eur. requirements are more stringent than the USP requirements nor does it mean that colistin might be much more active than polymyxin. The minimal potency limits indicated have only a relative value and the potency has to be determined against the appropriate reference substance; since all four reference substances are different, four different unit systems are involved. The difference in the Ph. Eur. limits for polymyxin and colistin arises because when the international unit of

*Presented at the "Fourth International Symposium on Drug Analysis", May 1992, Liège, Belgium.

colistin was established, the close relationship of this antibiotic with the polymyxins was not known.

Neomycin sulphate (mainly neomycin B) of the Ph. Eur. must contain 3–15% of neomycin C and the minimal limit for the potency is 680 IU mg^{-1}. The USP limit is 600 $\mu g\ mg^{-1}$. These limits do not indicate that the Ph. Eur. is more stringent that the USP. Indeed, in each assay a different reference substance has to be used. Framycetin sulphate (almost pure neomycin B) of the Ph. Eur. must contain $\leq$3% of neomycin C and the limit for the potency is 630 IU mg^{-1}. This low value is due to the establishment of a different international standard for framycetin. Neomycin C is less active than neomycin B. This situation could be criticised but the decisions of the WHO committees have to be accepted. The USP does not describe framycetin.

Microbiological Assay and Implications for the Content of Preparations

Except for benzylpenicillin and its salts (e.g. with benzathine or procaine), no generally accepted relationship exists between the potency (IU mg^{-1}) and the content (% w/w); 1 mg of benzylpenicillin sodium is equivalent to 1670 IU mg^{-1} [5] and to 1667 U mg^{-1} (USP). The theoretical potency of the other salts can be calculated on the basis of molecular weight. For other antibiotics, no agreement exists concerning the number of IU corresponding to the pure compound.

The fact that the content of an antibiotic is expressed as the potency or as % w/w may affect the content of preparations. For example, it can be stated that a preparation labelled to contain 5 mg of neomycin sulphate per dosage unit must contain at least 3400 IU (Ph. Eur. potency limit 680 IU mg^{-1} × 5); however, it may contain more IU if a bulk material of better than minimal quality has been used. When the labelled content is expressed as 3400 IU per dosage unit, it means that at least 5 mg of neomycin sulphate of minimal potency is present or that less than 5 mg is present, if a bulk material of better quality has been used. At the end of the shelf-life of a preparation with labelled content 3400 IU and registered with 95–105% limits, the minimal potency should correspond to at least 3230 IU per dosage unit. This also implies that the potency immediately after preparation should not exceed 3570 IU per dosage unit; thus the preparation may not be prepared with 5 mg of neomycin with potency >714 IU mg^{-1}. However, the Ph. Eur. chemical reference substance (Ph. Eur. CRS) has a potency of 775 IU mg^{-1}, calculated on an 'as is' basis, which indicates that bulk products with potency >714 IU mg^{-1} (dry substance) may exist on the market. The problem with these preparations is that during manufacturing their content cannot be adapted to that limit, obtained if 100% pure bulk product has been used, simply because the potency corresponding to 100% pure substance is not officially stated.

The situation is different for preparations made with bulk products, the content of which is expressed in % w/w. Consider a preparation of benzylpenicillin sodium (Ph. Eur. limits: 96.0–100.5% w/w) labelled to contain 600 mg of benzylpenicillin sodium per dosage unit. For this preparation an amount of bulk product corresponding to 600 mg × (100/% w/w content) is used. For registration of the preparation with 95–105% limits, the content at the end of the shelf-life must correspond to 570 mg of pure benzylpenicillin sodium.

These examples show that in a preparation, less of the antibiotic has to be present when the assay is performed by microbiological assay than when the assay is performed with a chemical or physicochemical method that permits expression of the content in % w/w. Indeed, for the example of the neomycin preparation it can be calculated, if it is accepted that the Ph. Eur. CRS (775 IU mg^{-1}) is 100% pure, that this difference is as much as 14%. In fact, the difference is even greater since in reality this Ph. Eur. CRS is not 100% pure. It is to be hoped that this is not a reason why some analysts continue to adhere vigorously to a microbiological assay.

Liquid Chromatography to Replace Microbiological Assay

Of course, a microbiological assay allows the determination of the total activity of a sample but it must be emphasized that the assay is performed in '*in vitro*' conditions and with a non-pathogenic strain [2]. Important disadvantages of a microbiological assay are its poor precision and therefore its poor accuracy, unless a large number of determinations are performed. In a recent collaborative study, performed in six laboratories to establish the

potency of the Ph. Eur. CRS 3 for erythromycin, the range (7%) covered by the means of the six laboratories was a measure for the between-laboratory precision (reproducibility); the 95% confidence limits (within-laboratory precision or repeatability) for one series of assays ranged from 907–933 IU mg^{-1} (turbidimetry) to 803–946 IU mg^{-1} (diffusion). These extreme values for the confidence limits were obtained in the same laboratory. In all, 63 of such series of assays were performed [6]. In a collaborative study, performed in 11 laboratories to determine the potency of the Ph. Eur. CRS for bacitracin zinc, the mean results were spread over a range of 17% [7].

As Vanderhaeghe [2] pointed out, it is clear that a microbiological assay with its selectivity towards antibiotically active molecules will continue to be of particular value for assaying complex mixtures such as bacitracin, colistin, nystatin or polymyxin. A chemical assay can be used where the chemical structure of the active component is established and where the sample is not a complex mixture of active components. The selectivity of the reaction can increase the value of the chemical assay. A nice example is the assay of β-lactam antibiotics by iodimetry which permits intact β-lactams to be distinguished from hydrolysed β-lactams. This needs two titrations and, since the stoichiometry of the reaction is not well understood, a reference substance has to be analysed simultaneously. Thus, four titrations are needed to obtain one assay value. In contrast, for the similarly selective mercurimetric titration of penicillins, now prescribed by the Ph. Eur., the stoichiometry is well established and no reference substance is needed. This allows the number of titrations to be halved. However, examples of selective chemical reactions with defined stoichiometry are not numerous.

Chromatography is a technique which can be highly selective but needs a reference substance because it is a comparative method. Gas chromatography is of limited value for quantitative analysis of antibiotics because most are not volatile or need a rather delicate derivatization [2]. Planar chromatography (TLC) is prescribed in pharmacopoeias mainly for qualitative or semi-quantitative use [8]. An important advantage of this technique is that the stationary phases are disposable so that experimental conditions can be more extreme and sample preparation can be simplified. In theory, all the components can be detected whereas in LC components can be retained on the column. Disadvantages of TLC are the price of the spectrodensitometric equipment, the somewhat poorer precision, problems with automation but above all the lack of published methods for antibiotics. It is generally accepted nowadays that LC and TLC are complementary but only a few validated methods for the quantitative analysis of antibiotics in bulk have been described in detail. Work in the author's laboratory with tetracyclines has confirmed the value of TLC [9–11].

LC is a potentially selective analytical method but its selectivity depends largely on the quality of the method development and validation and also on the quality of the stationary phase which, for most reversed-phases, varies as a function of age. The repeatability depends much on the equipment and the analyst. The importance of proper peak integration is often overlooked. The equipment and the analyst play also an important role in the reproducibility of the method but the stationary phase is highly important. Within a company, a defined brand or even a defined batch of material can be used. However, for a pharmacopoeial method, only general specifications of the column are specified. Therefore it is important, especially for official methods, that the applicability of the method on several stationary phases is examined during validation and that a system suitability test is developed. Methods giving satisfactory separations with only one brand of stationary phase, which is difficult to characterize in general terms, should be avoided. Optimally, a system suitability test should verify the proper separation of those components of the substance to be examined, which are difficult to separate. A system suitability test not only helps to select the proper brand of stationary phase but it is also possible to check whether a column, which previously gave good separations in a given method, is still in good working order. Indeed, reversed-phase stationary phases are not stable and their properties change upon use.

To avoid problems of poor reproducibility due to variability in the selectivity of silica-based reversed-phases, consideration might be given to the use of a stationary phase which is more stable and whose chromatographic performance is less dependent upon its origin. Poly(styrene-divinylbenzene) (PSDVB) is such a stationary phase which is very stable over

long periods of use and in extreme conditions of pH (1–13) and which has been observed to cause no problems of variability in selectivity and reproducibility due to differences in origin or in age of the stationary phase [12–14]. In the following section a few examples are discussed where PSDVB was found to be a good replacement for silica-based stationary phases in the analysis of antibiotics.

Poly(Styrene-Divinylbenzene) as a Stationary Phase for the Analysis of Antibiotics

Erythromycin

In a previous paper on liquid chromatography some problems with the reversed-phase analysis of erythromycin have been discussed [15]. It was concluded that the separation of erythromycin E (EE) from the main component erythromycin A (EA) is the most difficult problem to be solved. It was also observed that ageing of the silica-based stationary phase allowed the improvement of the separation of EA from its related substances [16, 17]. A typical chromatogram is shown in Fig. 1. The structures of erythromycin and of its related substances are shown in detail elsewhere [18].

A column-switching technique was developed to improve the separation and to reduce the analysis time [18]. A typical chromatogram is shown in Fig. 2. Although this technique was

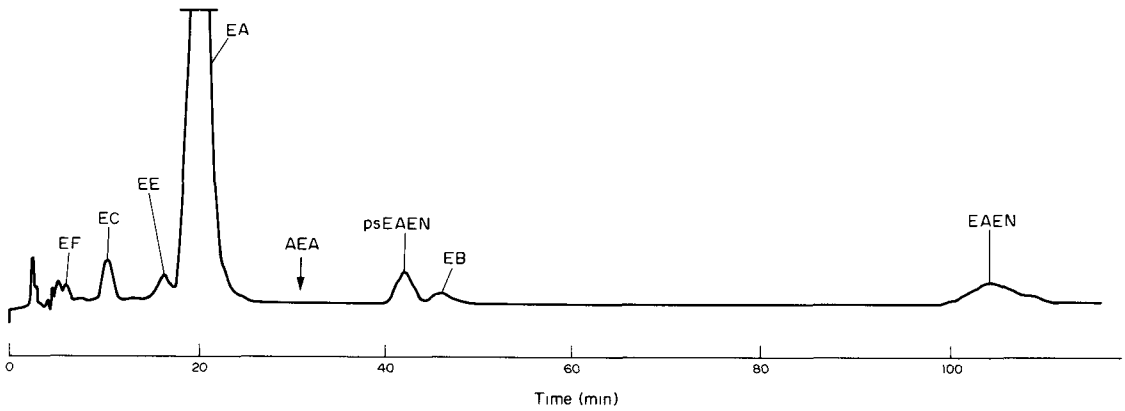

Figure 1
Typical chromatogram of a commercial erythromycin sample. Stationary phase: aged 10-μm RSil C18 LL in a 250 × 4.6 mm i.d. column. Mobile phase: acetonitrile–tetrabutylammonium sulphate (pH 6.0, 0.2 M)–ammonium phosphate buffer (pH 6.0, 0.2 M)–water (23:5:5:67, v/v/v/v). Temperature: 35°C, flow rate: 1.5 ml min^{-1}, detection: UV at 210 nm. Sample injected: 200 μg. EF = erythromycin F, EC =erythromycin C, EE = erythromycin E, EA = erythromycin A, AEA = anhydroerythromycin A, psEAEN = pseudo-erythromycin A enol ether, EB = erythromycin B and EAEN = erythromycin A enol ether.

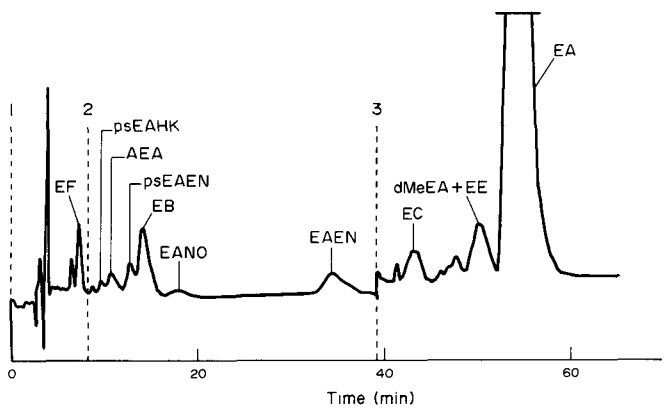

Figure 2
Typical chromatogram of a commercial erythromycin sample obtained with a column-switching technique. Columns: 75 × 4.6 mm i.d. and 250 × 4.6 mm i.d. See Fig. 1 for stationary phase and other experimental conditions. Between 1 and 2 substances were eluted through the two columns. Between 2 and 3 substances were eluted through the short column only; meanwhile the other substances were blocked in the longer column; these were finally eluted after 3. psEAHK = pseudo-erythromycin A hemiketal, EANO = erythromycin A *N*-oxide, dMeEA = demethylerythromycin A. See Fig. 1 for other abbreviations.

routinely used in the author's laboratory it was felt that it was too laborious to be set up for occasional analysis. Important differences in the selectivity of the stationary phase were observed also and therefore the technique was found unsuitable as a general method. Finally a method using PSDVD was developed [19].

A much better separation was obtained on wide pore material (1000 Å) than on 100-Å material. A typical chromatogram is shown in Fig. 3. The pH of the mobile phase is pH 9.0, which, for reasons of stability, cannot be used with silica-based stationary phases, certainly not at 70°C. Heating improves the efficiency and reduces the back-pressure. The efficiency obtained with this system is only about 5000 plates m^{-1}; therefore the quality of the separation mainly relies upon good selectivity. This LC method was examined in a collaborative study [J. Paesen *et al.*, in preparation] carried out in six laboratories on seven different columns. It was observed that the selectivity obtained in the different laboratories was comparable. Some laboratories had problems in achieving adequate heating of the column, especially when a flow rate of 2.0 ml min^{-1} was used. These technical problems are discussed elsewhere [20]. The repeatability and reproducibility of the LC method for erythromycin, expressed as the relative standard deviation (RSD) of the results for the determination of erythromycin A, were 1.1 and 1.3%, respectively. This LC method has been proposed for use in the European Pharmacopoeia.

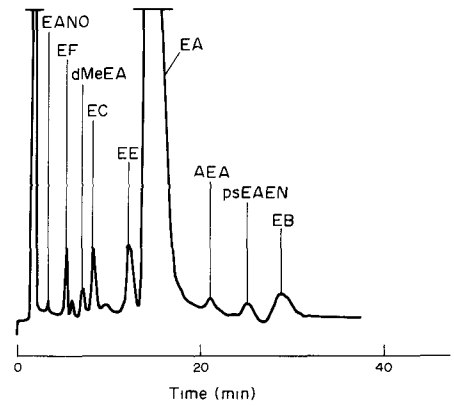

Figure 3
Chromatograms of a spiked sample of erythromycin. Stationary phase: 8-μm PLRP-S, 1000 Å. Mobile phase: acetonitrile–2-methyl-2-propanol–phosphate buffer (pH 9.0, 0.2 M)–water (3:16.5:5:75.5, v/v/v/v). Temperature: 70°C, flow rate: 2 ml min^{-1}, detection: UV at 215 nm. Sample injected: 400 μg. See Figs 1 and 2 for abbreviations.

Minocycline

LC using PSDVB has been successfully applied to the separation of tetracyclines. A method for the analysis of doxycycline has been published in the European Pharmacopoeia [3]. An analogous method for oxytetracycline has been adopted by the European Pharmacopoeia Commission. Similar methods for the analysis of tetracycline and demeclocycline are under study for the Ph. Eur. For minocycline, a LC method using a silica-based C8 reversed-phase is described by the USP [4], the British Pharmacopoeia (BP) [21] and the French Pharmacopoeia (Ph. Fr.) [22]. The main difference between these pharmacopoeial methods is the pH of the mobile phase. The USP prescribes a final pH of 6.0–7.0, the BP 6.2–6.5 and the Ph. Fr. 7.0 ± 0.05. The mobile phase contains dimethylformamide (DMF) as the organic modifier and also ammonium oxalate and sodium edetate. The applicability of the method on different C8 reversed-phase columns was examined [23]. The selectivity obtained on the stationary phases was found to be pH-dependent. This is probably an explanation why different pharmacopoeias prescribe different pH limits for the mobile phase. In general, mobile phases at higher pH gave the better separations. Besides the disadvantage that for each stationary phase the optimal pH has to be determined by the analyst, the method suffers from other inconveniences; for example, limited miscibility of the components of the mobile phase do not allow the DMF content to be increased freely in order to reduce the analysis time. This also excludes the use of C18 reversed-phases. The presence of DMF in the mobile phase causes background absorption and reduces the sensitivity, even when detection is performed at 280 nm. The most important disadvantage observed was the poor stability of the stationary phase, probably due to the presence of DMF and salts.

Therefore, a method was developed using PSDVB as the stationary phase [24]. A typical chromatogram is shown in Fig. 4. In addition to the very good selectivity which enables all known potential impurities and several of unknown identity to be separated, the method has the advantage that it is applicable on stationary phases of different origin and age.

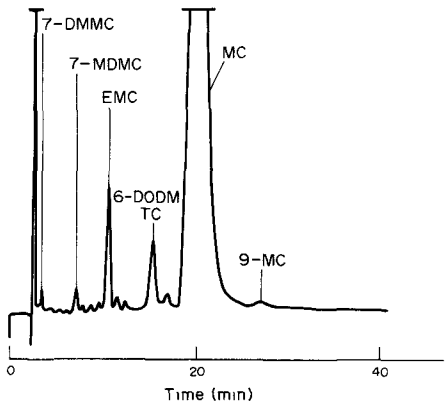

Figure 4
Chromatogram of an old sample of minocycline hydrochloride. Stationary phase: 8-μm PLRPS, 100 Å in a 250 × 4.6 mm i.d. column. Mobile phase: 2-methyl-2-propanol–phosphate buffer (pH 10.5, 0.2 M)–tetrabutylammonium sulphate (pH 10.5, 0.2 M)–sodium edetate (pH 10.5, 0.01 M)–water (7:10:10:10:63, m/v/v/v/v). Temperature: 60°C, flow rate: 1.0 ml min^{-1}, detection: UV at 254 nm. MC = minocycline, 7-DMMC = 7-didemethylminocycline, 7-MDMC = 7-monodemethylminocycline, EMC = 4-epiminocycline, 6-DODMTC = 6-deoxy-6-demethyl-tetracycline, 9-MC = 9-minocycline.

Conclusions

The existence of several systems to express the potency of antibiotics is a source of confusion and errors. The expression of the content in % w/w, which can be introduced for most antibiotics, would remove many problems and open the way to harmonization. This needs the introduction of physico-chemical methods such as LC. A major disadvantage of LC is the influence of the silica-based reversed-phase on the reproducibility. This can be avoided by the use of more stable and more uniformly performing stationary phases such as poly(styrene-divinylbenzene).

Acknowledgement — Mrs A. Decoux is acknowledged for secretarial assistance.

References

[1] A.H. Thomas, *J. Pharm. Biomed. Anal.* **5**, 319–324 (1987).
[2] H. Vanderhaeghe, *J. Pharm. Biomed. Anal.* **7**, 127–128 (1989).
[3] *European Pharmacopoeia*, 2nd edn. Maisonneuve, Sainte Ruffine, France (1980).
[4] *United States Pharmacopeia XXII.* The United States Pharmacopeial Convention, Rockville, MD, USA (1989).
[5] J.H. Humphrey, M.V. Musset and W.L.M. Perry, *Bull. WHO* **9**, 15–28 (1953).
[6] C. Pascal, personal communication.
[7] C. Pascal, *Pharmeuropa* **2**, special issue, 125–139 (1990).
[8] J. Sherma, *J. Assoc. Off. Anal. Chem.* **74**, 435–437 (1991).
[9] Weng Naidong, S. Geelen, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **8**, 891–898 (1990).
[10] Weng Naidong, C. Hauglustaine, E. Roets and J. Hoogmartens, *J. Planar Chromatogr.* **4**, 63–68 (1991).
[11] Weng Naidong, Sun Hua, K. Verresen, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **9**, 717–723 (1991).
[12] J.V. Dawkins, L.L. Lloyd and F.P. Warner, *J. Chromatogr.* **352**, 157–167 (1986).
[13] J. Hoogmartens, Naeem Hasan Khan, H. Vanderhaeghe, A.L. van der Leeden, M. Oosterbaan, G.L. Veld-Tulp, W. Plugge, C. van der Vlies, D. Mialanne, R. Melamed and J.H.McB. Miller, *J. Pharm. Biomed. Anal.* **7**, 601–610 (1989).
[14] J. Hoogmartens, Weng Naidong, Naeem Hasan Khan, A. Malley, U. Hearty, R. Melamed, J.P. Gousset, P. Creed, C. Woollam, J.H.McB. Miller, J. Fuchs and H. Vanderhaeghe, *Pharmeuropa* **2**, 77–85 (1990).
[15] J. Hoogmartens, *Pharmeuropa* **2**, special issue, 89–105 (1990).
[16] Th. Cachet, I. Quintens, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* **12**, 2171–2201 (1989).
[17] Th. Cachet, I. Quintens, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* **14**, 1203–1218 (1991).
[18] Th. Chachet, K. De Turck, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **9**, 547–555 (1991).
[19] J. Paesen, E. Roets and J. Hoogmartens, *Chromatographia* **32**, 162–166 (1991).
[20] J. Paesen and J. Hoogmartens, *LC–GC* **10**, 364–366 (1992), *LC–GC Intl.* **5–6**, 18–20 (1992).
[21] *British Pharmacopoeia.* HMSO, London (1988).
[22] *Pharmacopée Française*, 10th edn. Maisonneuve, Sainte Ruffine, France (1992).
[23] Weng Naidong, K. Vermeulen, I. Quintens, E. Roets and J. Hoogmartens, *Chromatographia* **33**, 560–566 (1992).
[24] Weng Naidong, J. Thuranira, K. Vermeulen, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, accepted.

[*Received for review 6 May 1992; revised manuscript received 3 June 1992*]