*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 9, Nos 10–12, pp. 829–833, 1991
Printed in Great Britain

0731-7085/91 $3.00 + 0.00
Pergamon Press plc

# Estimation of impurity profiles of drugs and related materials. Part VIII: combined application of high-performance liquid chromatography and NMR spectroscopy in the impurity profiling of drugs*†

S. GÖRÖG, G. BALOGH and M. GAZDAG

*Chemical Works of Gedeon Richter Ltd, P.O.B. 27, H-1475, Budapest, Hungary*

**Abstract**: The usefulness of the joint application of HPLC and NMR spectroscopy in drug impurity profiling is demonstrated by the following examples: (1) identification of *Z* and *E* isomers of 17α-ethynyl-4-oestrene-3β,17-diol-3-acetate-17-(3′-acetoxy-2′-butenoate) in ethynodiol diacetate; (2) identification of the *p*-tolyl analogue as the impurity of enalapril maleate; (3) identification and quantification of 2′-dehydro-pipecuronium bromide in pipecuronium bromide. The possibilities of utilizing NMR spectroscopy for the identification and quantification of the impurities with and without their isolation are discussed.

**Keywords**: *HPLC; NMR spectroscopy; drug impurity profiling; ethynodiol diacetate; enalapril maleate; pipecuronium bromide.*

## Introduction

In the previous publications from the authors' laboratory dealing with impurity profiling of drugs and related materials the separation method was usually HPLC [2–12], or in some cases gas chromatography [1, 2, 9, 13, 14]. Of the spectroscopic techniques used in conjunction with the chromatographic methods the application of diode-array UV spectroscopy is emphasized in some cases [7, 9, 11], while in many others the complex application of a variety of spectroscopic methods has been demonstrated [1, 2, 5, 8, 12, 14]. In this paper three examples are presented aiming at demonstrating the high potential of NMR spectroscopy in conjunction with HPLC in drug impurity profiling. Although the drawback of NMR spectroscopy in comparison with mass spectrometry is that the sample size required for structure elucidation of an impurity is much higher. Moreover, unlike mass spectrometry, no commercially available on-line HPLC–NMR instruments are accessible, although the advantages are the almost universal capability of high-resolution NMR spectroscopy in structure elucidation, coupled with the possibility of obtaining useful information without preliminary isolation of the impurity, together with the possibility of using this technique as a useful tool for the quantification of impurities. The three examples in this paper have been selected in such a way that all the above described advantageous features of the combined use of HPLC and NMR spectroscopy can be demonstrated.

## Experimental

The HPLC separations were carried out with a Hewlett–Packard 1090A chromatograph equipped with a HP 1040 diode-array UV detector, and also with a Waters 600E Multisolvent Delivery System equipped with 990 diode-array UV detector, NEC/APC IV computer and U6K variable-volume injector. For the chromatographic conditions see the following text.

The NMR spectra were recorded on a Varian VXR-300 or on a XLAA-400 instrument, respectively.

### *Identification of* Z *and* E *isomers of 17α-ethynyl-4-oestrene-3β, 17-diol-3-acetate-17-(3′-acetoxy-2′-butenoate) (I and II) in ethynodiol diacetate (EDDA)*

The separation factors of two isomeric impurities I and II from EDDA are $\alpha_{I/EDDA}$ =

* Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

† For Part VII see ref. 1. This paper is Part XLIII in a series on "Analysis of Steroids"; for Part XLII see ref. 2.

0.94 and $\alpha_{II/EDDA}$ = 1.48, respectively, applying the reversed-phase system ($C_{18}$ column and methanol–water; 7:3, v/v) used in the course of earlier studies on the estimation of the impurity profile of EDDA [3, 12]. Because of the poor resolution of I from EDDA and the necessity of using preparative HPLC for the isolation of the impurities for structure elucidation, the system was changed to a normal phase system based on LiChrosorb SI-100 with a highly volatile eluent (98:2, v/v mixture of hexane–2-propanol). In this system the separation factors are $\alpha_{I/EDDA}$ = 1.84 and $\alpha_{II/EDDA}$ = 1.21, respectively.

Because of the limited value of the information obtained from the diode-array UV spectra (slight bathochromic shift and broadening of the band attributable to the double bond in EDDA) and from the mass spectra (lack of molecular ion; fragment peak at *m/z* 438 indicating the presence of additional acetyl group(s)) the NMR spectra of the isolated impurities were taken as the basis for structure elucidation.

Figure 1 shows the reaction scheme of the final step of the synthesis of EDDA and the side reactions causing the formation of impurities I and II, together with the chemical shifts observed in their NMR spectra. The signals of protons and carbon atoms other than those in the 17-*O*-acyl moiety are almost exactly the same in the spectra of all three compounds. The identification of the *Z* and *E* isomers was made on the basis of the anisotropic shielding effect of the carbonyl group of the 3′-acetoxy group.

In I and II the 17-*O*-acetyl group of EDDA is replaced by the 3′-acetoxy-2′-butenoate group, which can be considered to be the 'trimerized' form of the acetyl group. The formation of this is due to the self-acetylation of acetic anhydride in the acetic anhydride–4-dimethylamino-pyridine complex used for the acetylation of ethynodiol [15].

*Identification of the* p-*tolyl analogue as an impurity in enalapril maleate*

During the HPLC investigation of crude enalapril maleate an impurity (III) was found in some batches: $\alpha_{III/enalapril}$ = 1.78 (Column: 250 × 4 mm; octylsilica, 5 μm (BST, Budapest, Hungary). Eluent: 1:1, v/v mixture of

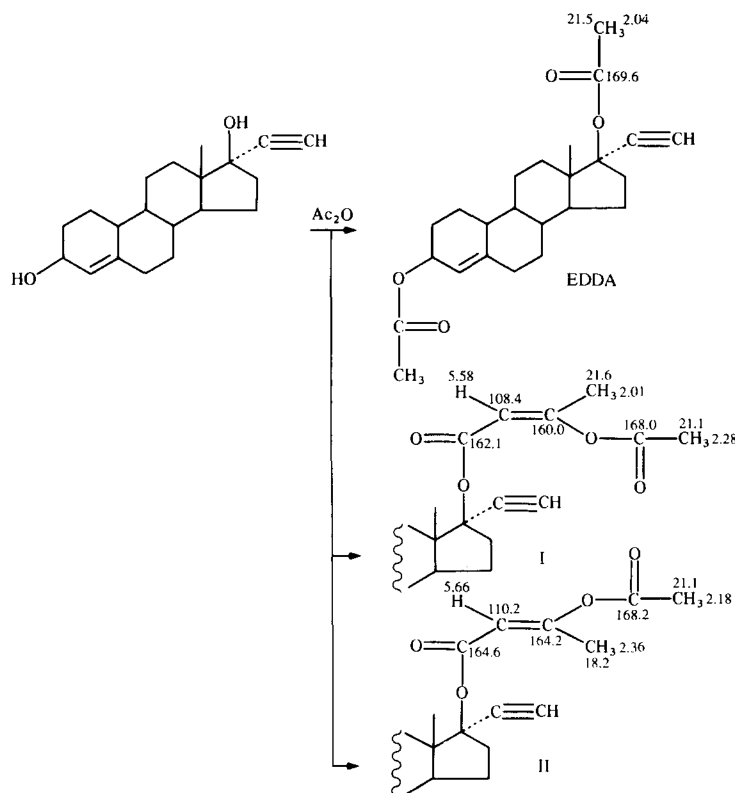


**Figure 1**
Reaction scheme of the final step of the synthesis of EDDA and two side reactions. The figures at the H and C atoms in the 17-*O*-acyl moiety are the chemical shifts in ppm in the $^1$H- and $^{13}$C-NMR spectra (solvent $CDCl_3$, reference TMS).

methanol–phosphate buffer; 0.04 M; pH = 2).

In the diode-array UV-spectrum of the impurity a remarkable bathochromic shift of the benzenoid band of the phenyl moiety was found relative to enalapril: a shift from 258 to 264 nm. On the basis of this, alkyl substitution in the phenyl ring was highly probable. This supposition was supported by the mass spectrum with its molecular ion higher by 14 units than that of enalapril, indicating that the substituent was likely to be a methyl group. NMR spectroscopy furnished evidence for the existence and location of the methyl group: a singlet at 2.26 ppm, a multiplet of aromatic protons of the 4-substituted benzene ring at 7.11 ppm.

It is worth mentioning that in this case it was not at all necessary to isolate the impurity to take the NMR spectra (Fig. 2): the high resolution NMR spectra of the crude samples enabled the above mentioned characteristic bands of III to be estimated if its quantity exceeded 1%. The final proof for the structure of III was its synthesis and subsequent retention matching with the impurity.

The reason for the formation of III is certainly the presence of toluene as an impurity of benzene, which is one of the starting materials of the synthesis of enalapril. On the basis of this information it has been easy to devise procedures to avoid the formation of this impurity.

*Identification and quantification of 2′-dehydro-pipecuronium bromide in pipecuronium bromide*

In the course of the purity tests by HPLC of the neuromuscular blocking agent pipecuronium bromide (ARDUAN®, Chemical Works of Gedeon Richter, Budapest, Hungary) [16] an impurity due to oxidative degradation was detected which was found to be the 2′-dehydro analogue (IV) of pipecuronium bromide.

The separation factor $\alpha_{\text{pipecuronium bromide/IV}}$ is 1.24 using silica methanol–acetonitrile–aqueous ammonia (43:43:14, v/v/v) containing 0.1 mole $l^{-1}$ of ammonium chloride and ammonium carbonate [17]. It was 1.54 in a normal phase ion-pairing system on silica with acetonitrile–water (96:4, v/v) containing 0.1 mole $l^{-1}$ sodium perchlorate [18].

In addition to the useful information obtained from the diode-array UV spectrum of IV with a maximum at 235 nm, characteristic of its enamine structure [19] and from the FAB mass spectrum also indicating the presence of

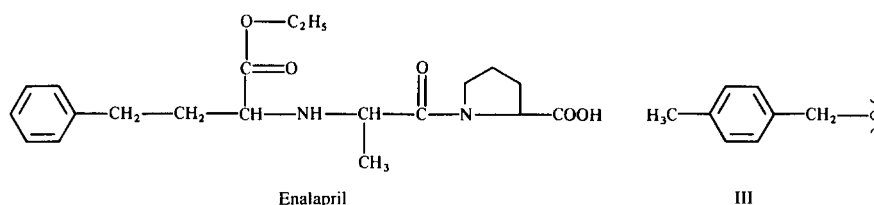


**Figure 2**
Formulae for enalapril and related impurity III.

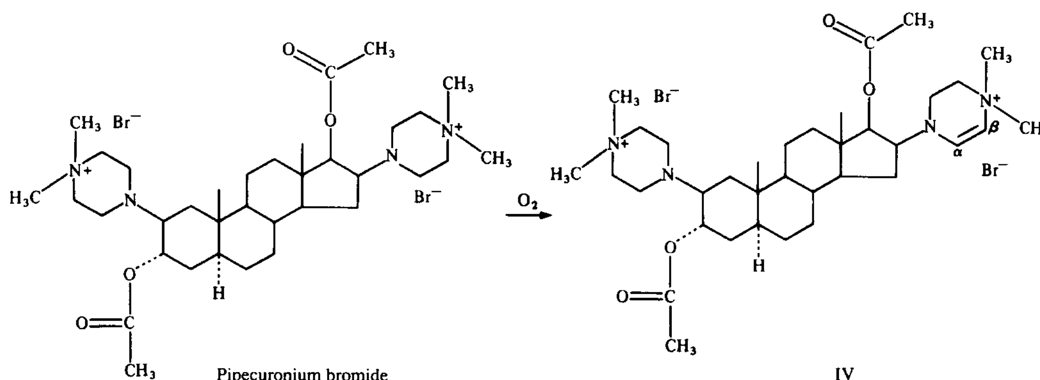


**Figure 3**
Oxidation of pipecuronium bromide to form IV.

one double bond in the molecule, NMR spectroscopy played a predominant role in the determination of the structure of the impurity.

Further to the signals of the vinylic protons in the 16β-methylpiperazinyl ring (doublets of $H_\alpha$ at 6.19 ppm; $J = 5.8$ Hz and $H_\beta$ at 5.26 ppm; $J = 5.8$ Hz) the following differences between the NMR spectra of pipecuronium bromide [20] and IV are characteristic of the location of the double bond: the quartet of H-16 which is at 3.24 ppm in the $^1$H-spectrum of pipecuronium bromide is shifted to 3.84 ppm, in the case of the doublet of H-17 the shift is from 4.78 to 4.68 ppm and even the singlet of the 18-methyl group is shifted from 0.79 to 0.84 ppm.

NMR spectroscopy played a predominant role also in establishing the basis for the quantification of IV in pipecuronium bromide by HPLC. As a consequence of the above mentioned enamine-type UV spectrum of IV, its absorbance at the UV detection wavelength (213 nm) is higher by almost one order of magnitude than that of pipecuronium bromide, where no strong chromophoric groups are present. For this reason the quantification of IV by area normalization would lead to erroneously high results. This could be avoided either by the external standard method or by using a correction factor taking into account the ratio of the detector responses of IV and pipecuronium bromide, but then a working standard of impurity IV would be necessary. As a consequence of the difficulties in isolating this minor and rather unstable impurity in pure form, this working standard (obtained by column chromatographic separation) was prepared as a concentrated, but not pure, form of IV. To determine its exact content of IV a quantitative method independent of HPLC was necessary. NMR spectroscopy with a suitable internal standard proved to be an excellent tool for this purpose. As the internal standard, 4-androstene-3,17-dione was selected making use of the singlet at 5.67 ppm of the H-4 signal in its NMR spectrum.

Figure 4 shows a section of the respective NMR spectra. As is seen in curve 'A', the only signal between 5 and 6.4 ppm in the spectrum of pipecuronium bromide is the broad peak of H-3 at 5.16 ppm. In the spectrum of IV (curve 'B') this appears at the same place. In this spectrum the doublets at 6.19 and 5.26 ppm are the signals of the vinylic protons $H_\alpha$ and $H_\beta$, while the additional singlet at 5.67 ppm in curve 'C' represents the above mentioned H-4 signal of the internal standard.

The concentration of IV as a percentage can be calculated using the following formula:

$$C_{IV} = \frac{100 m_{AD}\, M_{IV}\, I_{IV}}{m_{sample}\, M_{AD}\, I_{AD}},$$

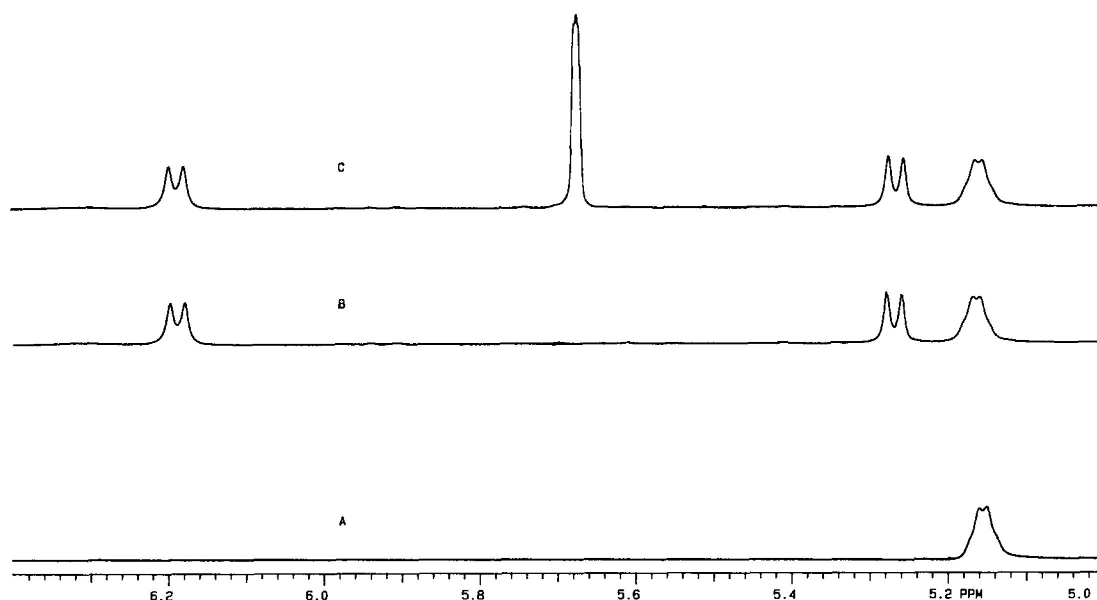


**Figure 4**
$^1$H-NMR spectra. Solvent: 2:1 (v/v) mixture of $CDCl_3$ and DMSO-$d_6$; reference: TMS. Curve A: 20.1 mg pipecuronium bromide per millilitre; curve B: 24.0 mg of the working standard of IV; curve C: 24.0 mg of the working standard of IV + 12.9 mg of 4-androstene-3,17-dione per millilitre.

where $m_{AD}$ and $m_{sample}$ are the weights of the internal standard and working standard, respectively, $M_{IV}$ and $M_{AD}$ are the molecular weights (760.71 and 286.42), $I_{IV}$ and $I_{AD}$ are the integrals of the signals at 5.26 or 6.19 ppm for IV and at 5.67 ppm for the internal standard, respectively. The RSD of the measurement was $\pm 4.1\%$ ($n = 5$).

It is interesting to note that using a concentration of 40 mg $ml^{-1}$ pipecuronium bromide and acquisition times in the range of 3.5 h, this method has been found to be suitable for the detection and quantification of IV in pipecuronium bromide down to the 0.5% (w/w) impurity level. This reference method has proved very useful in the course of validating the HPLC method for the determination of IV in pipecuronium bromide. The details of this method will be the subject of another publication.

## Conclusions

The three examples presented in this paper represent three different levels of the use of NMR spectroscopy in conjunction with HPLC in drug impurity profiling.

In the most common cases the sequence of steps is: analytical HPLC; preparative HPLC; and identification of the isolated impurities by spectroscopic (mainly NMR) methods, as exemplified by the estimation of I and II in EDDA.

In advantageous cases the second step can be omitted: the NMR spectrum of the contaminated bulk drug is suitable for the detection and identification of the impurity (cf. the identification of the tolyl analogue (III) in enalapril and to some extent the detection of the dehydro analogue (IV) in pipecuronium bromide).

In addition to these possibilities, in some cases NMR spectroscopy can be a useful tool in the quantification of impurities, as shown in the case of dehydro-pipecuronium bromide (IV) in pipecuronium bromide.

## References


[1] A. Laukó, É. Csizér and S. Görög, *J. High Res. Chromatogr.*, in press.
[2] A. Laukó, A. Csehi, G. Balogh, É. Csizér, B. Herényi and S. Görög, *Acta Pharm. Hung.* **61**, 98–104 (1991).
[3] S. Görög and B. Herényi, *J. Chromatogr.* **152**, 240–242 (1978).
[4] B. Herényi and S. Görög, *Acta Pharm. Hung.* **50**, 173–176 (1980).
[5] S. Görög, B. Herényi and M. Lõw, *J. Chromatogr.* **353**, 417–424 (1986).
[6] S. Görög, B. Herényi and É. Csizér, *Acta Chim. Hung.* **122**, 251–259 (1986).
[7] S. Görög and B. Herényi, *J. Chromatogr.* **400**, 177–186 (1987).
[8] S. Görög, A. Csehi, B. Herényi, M. Bihari, É. Csizér and A. Laukó, *Acta Pharm. Hung.* **57**, 35–43 (1987).
[9] S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988).
[10] S. Görög, B. Herényi, O. Nyéki, I. Schön and L. Kisfaludy, *J. Chromatogr.* **452**, 317–321 (1988).
[11] S. Görög, M. Rényei and B. Herényi, *J. Pharm. Biomed. Anal.* **7**, 1527–1533 (1989).
[12] S. Görög, Zs. Halmos, B. Herényi, A. Georgakis, G. Balogh, É. Csizér and Z. Tuba, in *Advances in Steroid Analysis '90* (S. Görög, Ed.), pp. 323–331. Akadémiai Kiadó, Budapest, 1991 (1991).
[13] A. Laukó and S. Görög, *J. Chromatogr.* **118**, 409–411 (1976).
[14] S. Görög, A. Laukó, B. Herényi, A. Georgakis, É. Csizér, G. Balogh, Gy. Gálik, S. Mahó and Z. Tuba, *Chromatographia* **26**, 316–320 (1988).
[15] G. Höfle, W. Steglich and H. Vorbrüggen, *Angew. Chem.* **90**, 602–615 (1978).
[16] Z. Tuba, *Arzneim. Forsch.* **30**, 342–346 (1980).
[17] G. Szepesi, M. Gazdag and K. Mihályfi, *J. Chromatogr.* **464**, 265–278 (1989).
[18] M. Gazdag, M. Babják, P. Kemenes-Bakos and S. Görög, *J. Chromatogr.*, **550**, 639–644.
[19] G. Opitz, H. Hellmann and H.W. Schubert, *Liebigs Ann. Chem.* **623**, 112–117 (I) 117–124 (II) (1959).
[20] S. Görög (Ed.), *Steroid Analysis in the Pharmaceutical Industry*, pp. 189–191. Ellis Horwood, Chichester (1989).


[*Received for review 29 April 1991; revised manuscript received 3 June 1991*]