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The use of capillary electrophoresis as part of a specificity testing strategy for mitoguazone dihydrochloride HPLC methods

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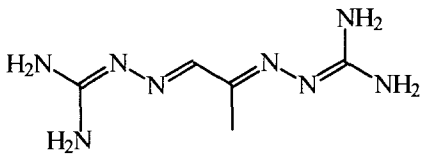
Abstract

Capillary electrophoresis (CE) has been used as part of a validation experiment designed to prove the specificity of high performance liquid chromatography (HPLC) methods used for analysis of mitoguazone dihydrochloride drug substance. Data regarding accuracy, precision and sensitivity of the CE methods are presented as well as a comparison of results obtained from CE, HPLC and thin-layer chromatography (TLC) analysis of samples stressed under a variety of conditions. It was concluded that, not only were the HPLC methods being investigated specific, but that CE could potentially be used to replace HPLC for the routine assay of mitoguazone dihydrochloride. © 1997 Elsevier Science B.V.

Keywords: Mitoguazone dihydrochloride; Specificity testing; Capillary electrophoresis; Reverse phase HPLC; Thin-layer chromatography; Validation

1. Introduction



.2HCl .H2O

(I)

Mitoguazone dihydrochloride (2,2′-(1-methyl-1,2-ethanediylidene) bis [hydrazinecarboxi-midamide] dihydrochloride hydrate (I)) is a cytotoxic, antineoplastic agent with demonstrated activity in a variety of tumour types. Although its mechanism of action is not fully understood, it is known to inhibit *S*-adenosylmethionine decarboxylase (SAMD), a key enzyme in polyamine synthesis, leading to cellular polyamine depletion [1]. Early clinical investigations with this material revealed unacceptable levels of toxicity; however, following the discovery that the levels of toxicity were dose related and could therefore be controlled, clinical interest revived [2,3].

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Even though mitoguazone dihydrochloride has been under consideration as a chemo-therapeutic agent for more than three decades, contemporary drug substance supplies must be controlled and stability tested using analytical methods validated to current internationally accepted standards [4]. Methods developed for analysis of mitoguazone dihydrochloride at our laboratory include reverse phase high performance liquid chromatography for the determination of its assay and chromatographic impurity level. As part of the validation of these methods, an indication of their level of specificity was required. For an assay, a high degree of specificity ensures that detection and quantitation of the analyte is not compromised by interference from species co-eluting with the main peak. When determining chromatographic impurity levels, a high degree of specificity provides confidence that all the potential species of interest are detectable. Where these species, including process impurities and degradation products, are not available to inject directly into the chromatographic system, specificity testing is conducted on stressed samples using one or more techniques which can usually be placed in one of two broad categories:

(a) Use of a specialised detection system to extract further information from the analyte peak.

(b) Some form of comparison between complimentary separation techniques, the first being the system under validation and the second being a system which, by virtue of its differing selectivity, might be expected to resolve species co-eluting in the first instance.

Examples of techniques in the first category include the use of diode array (DAD) [5] and mass spectroscopy [6] detectors to obtain UV/visible or mass spectra respectively from various positions through the analyte peak, potentially allowing the detection of co-eluting species. Techniques in the second category include the use of flow switching apparatus to divert the analyte peak onto a second stationary phase with a different selectivity, or comparison of results from the system being validated with those from a second chromatographic technique such as thin-layer chromatography (TLC). It is in this context that the use of capillary electrophoresis (CE)is described as part of an high performance liquid chromatography (HPLC)/DAD/CE/TLC specificity testing strategy for the mitoguazone dihydrochloride methods.

2. Experimental

2.1. Equipment and chemicals

CE data were generated on a BioFocus™ 3000 system (Bio-Rad Laboratories, Hemel Hempstead, Herts). HPLC data were generated using various Kontron (Watford, Herts) and Waters (Watford, Herts) pump, autosampler, column oven and detector models. HPLC and CE data were processed using a Multichrom™ V1.8-2 (LabSystems, Altringham, Cheshire). UV/visible absorption spectra were captured using an HP 1040 diode array detector (Hewlett Packard, Bracknell, Berks.). Light stressing (Xenon source, filtered through window glass) was performed in a Haraeus Suntest™ apparatus (Alplas Technology, Oxford). Acetonitrile and heptanesulphonic acid (sodium salt), were HPLC grade. Inorganic chemicals and ACVA (4,4′-azobis(4-cyanovaleric acid), a radical initiator, exposure to which mimics oxidative stress, were analytical reagent grade. Mitoguazone dihydrochloride and purified water were obtained inhouse.

2.2. Stress sample preparation

Samples of mitoguazone dihydrochloride (approximately 370 mg, equivalent to 250 mg base) were accurately weighed into 50 ml volumetric flasks and stressed according to the conditions given below. Stressing was continued for a maximum of 7 days or until 20–50% degradation had been achieved. After stressing, samples were neutralised, if necessary, and diluted to volume with purified water to give ~ 5 mg(base) ml^{-1} solutions for TLC analysis. Aliquots of these solutions were diluted with purified water to give ~ 1 mg(base) ml^{-1} solutions for use in impurity determinations by HPLC and CE. Finally, aliquots of these solutions were either diluted in HPLC mobile phase to give ~ 0.01 mg(base) ml^{-1} solutions

Table 1
Recovery data for CE and HPLC assays

Sample identity	CE data		HPLC data	
	Nominal added (%)	Average of nominal recovered[a] (%)	Nominal added (%)	Average of nominal recovered[b] (%)
Blank	0	0	0	0
80%	81.9	81.8	81.1	81.0
100%	103.2	103.2	103.2	103.1
120%	121.1	118.1	121.1	120.9

[a] Least squares regression analysis of the data gave an average accuracy of 99.1% (coefficient of correlation 0.989).
[b] Least squares regression analysis of the data gave an average accuracy of 99.8% (coefficient of correlation 0.999).

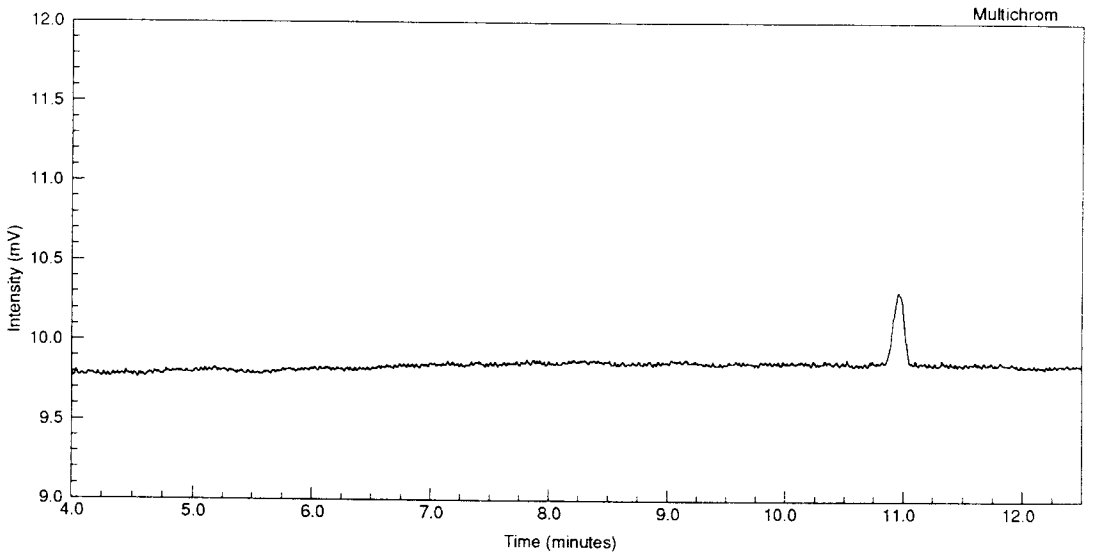

Fig. 1. Electropherogram for 0.1% w/w mitoguazone dihydrochloride sample

for HPLC assay or in 20 mM potassium dihydrogen phosphate, pH 3.0, to give ~0.025 mg(base ml^{-1} solutions for CE assay.

2.3. Stress conditions

Heat: sample held at 80°C for 7 days. Acidic: 10 ml of 0.1 M hydrochloric acid was added to the sample and the solution held at 70°C for 7 days. Basic: 10 ml of 0.1 M sodium hydroxide was added to the sample and the solution held at 70°C for 2 days.

Aqueous: 10 ml of purified water was added to the sample and the solution held at 70°C for 7 days. Oxidative: 10 ml of a 0.1 M aqueous ACVA solution was added and the sample held at 40°C for 7 days. Light: Sample received an overall illumination of ~15000 klux hours (with associated UV).

2.4. HPLC methods

2.4.1. Assay

Samples were chromatographed isocratically on a 25 × 0.46 cm i.d. Hypersil BDS C8 5 μm column (Anachem, Luton, Beds) using a mobile phase consisting of A: 0.05 M potassium dihydrogen orthophosphate buffer containing 1 g l^{-1} of hep-

Table 2
Assay and impurity data for stressed mitoguazone dihydrochloride

Stress condition	CE assays (% w/w)[a]	Impurities by CE (% w/w)[a]	HPLC assays (% w/w)[a]	Impurities by HPLC (% w/w)[a]	Impurities by TLC (% w/w)[a]
Control	100.0, 100.4, 100.1	1.5	100.6, 99.9	1.3	<0.7
Heat	102.2, 101.5, 102.8	1.4	100.1, 100.2	1.1	<1.1
Acidic	86.4, 88.5, 88.1	11.3	87.6, 87.0	14.0	<12.5
Basic	77.0, 75.1, 76.6	25.6	74.9, 75.4	27.9	<25.3
Aqueous	72.1, 71.1, 72.6	27.8	69.4, 69.1	31.9	<29.5
Oxidative	95.7, 94.6, 94.4	4.9	93.7, 94.5	6.5	<5.8
Light	97.1, 98.3, 98.6	1.3	99.9, 99.2	1.4	<0.7

[a] Results are expressed at % w/w of the initial base concentration.

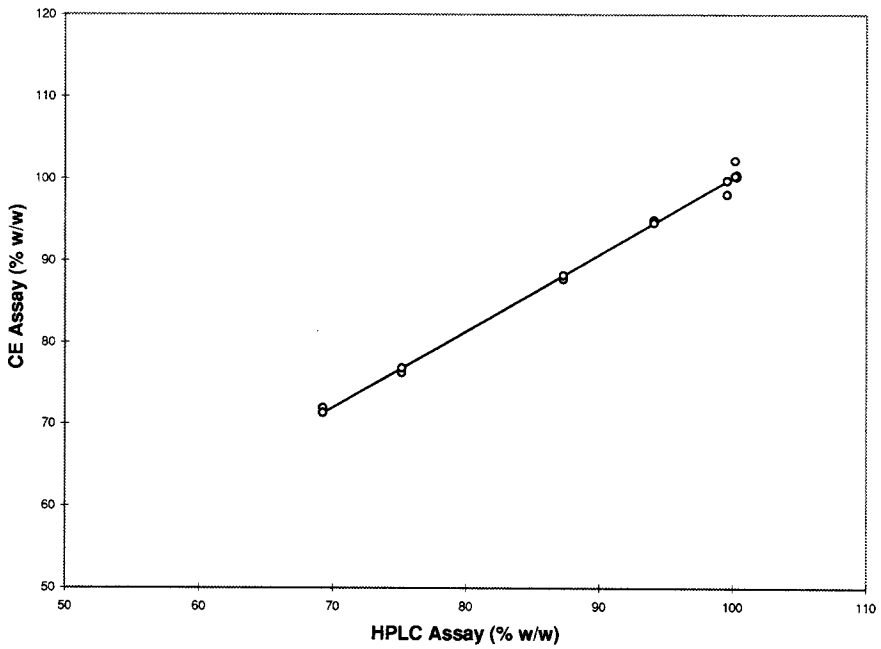

Fig. 2. Correlation between CE and HPLC assay results (coefficient of correlation 0.995)

tanesulphonic acid (sodium salt) and adjusted to ph 3.0 with concentrated orthophosphoric acid (89% by volume); and B: acetonitrile (89:11, v/v). The flow rate was 2 ml min^{-1}, the detector wavelength was 283 nm, the injection volume was 20 μl and the column temperature was 40°C. Samples were quantified with respect to an accurately prepared external standard (nominally 0.01 mg(base) ml^{-1}).

2.4.2. Impurity method

Chromatographic conditions were as for the assay except that a detector wavelength of 210 nm was used. A second HPLC system was used with a modified buffer to acetonitrile mobile phase ratio (85:15, v/v), primarily to estimate specific process impurities. Although the data presented below include a small contribution from impurities measured in this system they are not discussed

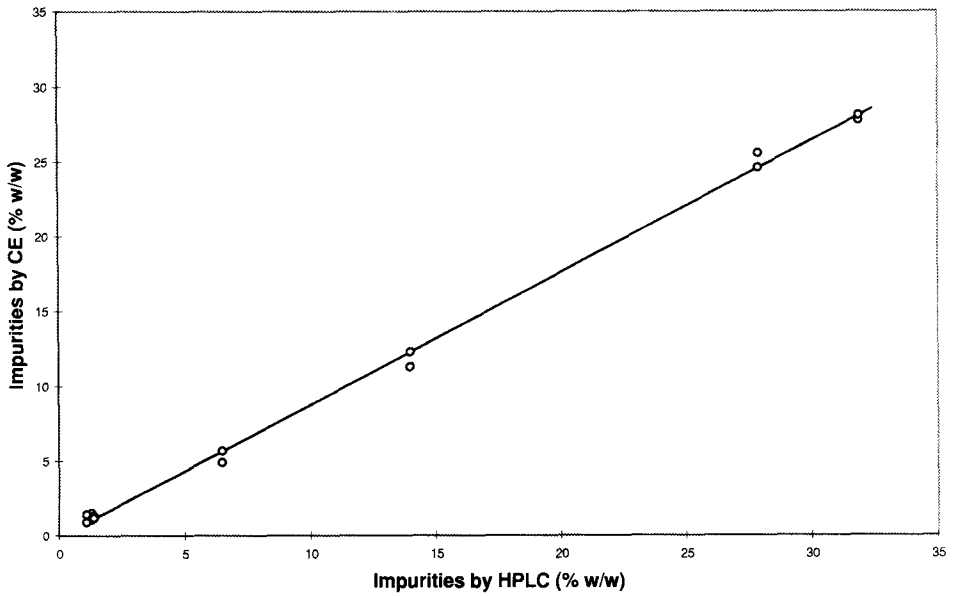

Fig. 3. Correlation between CE and HPLC impurity data (coefficient of correlation 0.998)

further. Impurities were quantified with respect to an accurately prepared external standard (nominally 0.005 mg(base) ml^{-1}).

2.5. CE methods

2.5.1. Assay

Electropherograms were generated on a 50 cm × 50 μm i.d capillary (Composite Metal Services, Worcester), conditioned with 1 M sodium hydroxide solution for 10 min followed by 0.1 M sodium hydroxide for 15 min and finally, water for 10 min. The run buffer was 0.1 M potassium dihydrogen orthophosphate buffer, pH 3.0. The pre-injection wash cycles were 0.1 M hydrochloric acid for 30 s and run buffer for 180 s. The applied voltage was +15 kV. The sample was injected hydrodynamically (5 psi for 4 s), the cartridge temperature was 23°C and the detector wavelength 283 nm. Samples were

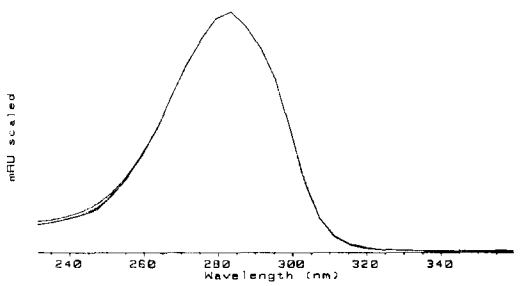

Fig. 4. Overlaid DAD spectra taken from the apex, upslope (approximately 10% of peak height) and downslope (approximately 10% of peak height) of the mitoguazone dihydrochloride HPLC peak from the sample stressed under acidic conditions

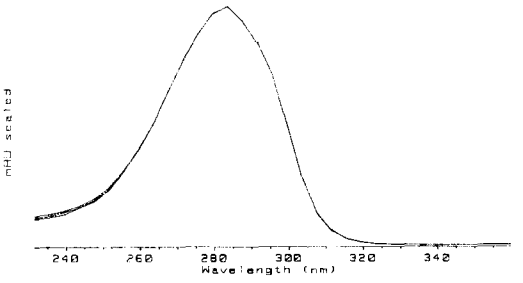

Fig. 5. Overlaid DAD spectra taken from the apex, upslope (approximately 10% of peak height) and downslope (approximately 10% of peak height) of the mitoguazone dihydrochloride HPLC peak from the sample stressed under basic conditions

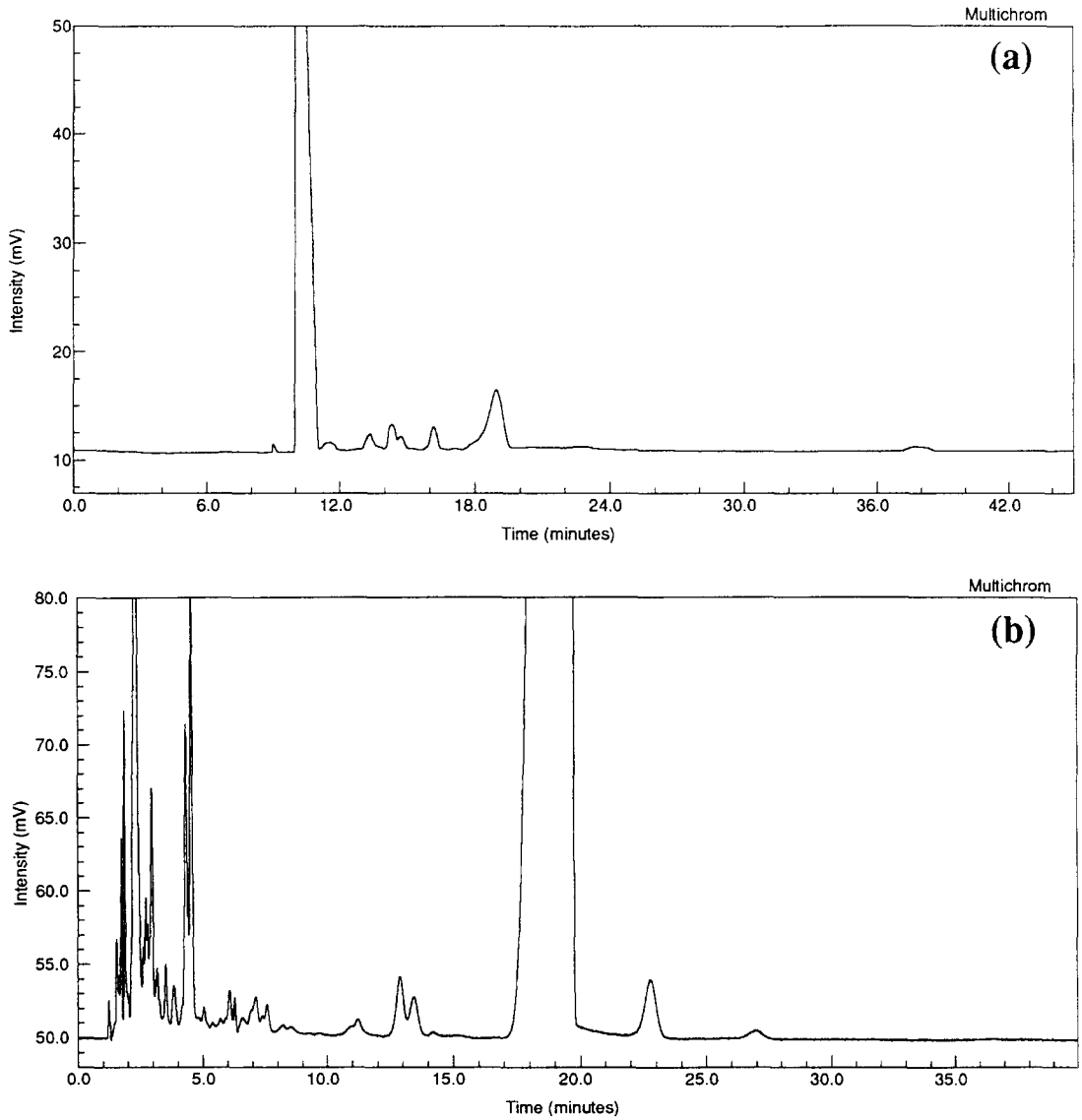

Fig. 6. CE electropherogram (a) and HPLC chromatogram (b) of sample stressed under acidic conditions

quantified with respect to an accurately prepared external standard (nominally 0.025 mg(-base) ml^{-1}).

2.5.2. Impurity method

Operating conditions were as for the assay, except that the detection wavelength was changed to 210 nm. Impurities (corrected for their migration times) were determined as a percentage of each electropherograms total area.

2.6. TLC method

20 μl of each sample was spotted onto a silica gel TLC plate (Merck 60F254). The plate was developed to a height of 10 cm in an acetone–ammonium hydroxide (SG 0.88)–water (90:5:5% by volume) mobile phase. Impurities were estimated against dilute mitoguazone dihydrochloride spots, both under short wavelength ultraviolet light (254 nm) and following treatment with a nitroprusside (sodium)-ferricyanide spray reagent [7].

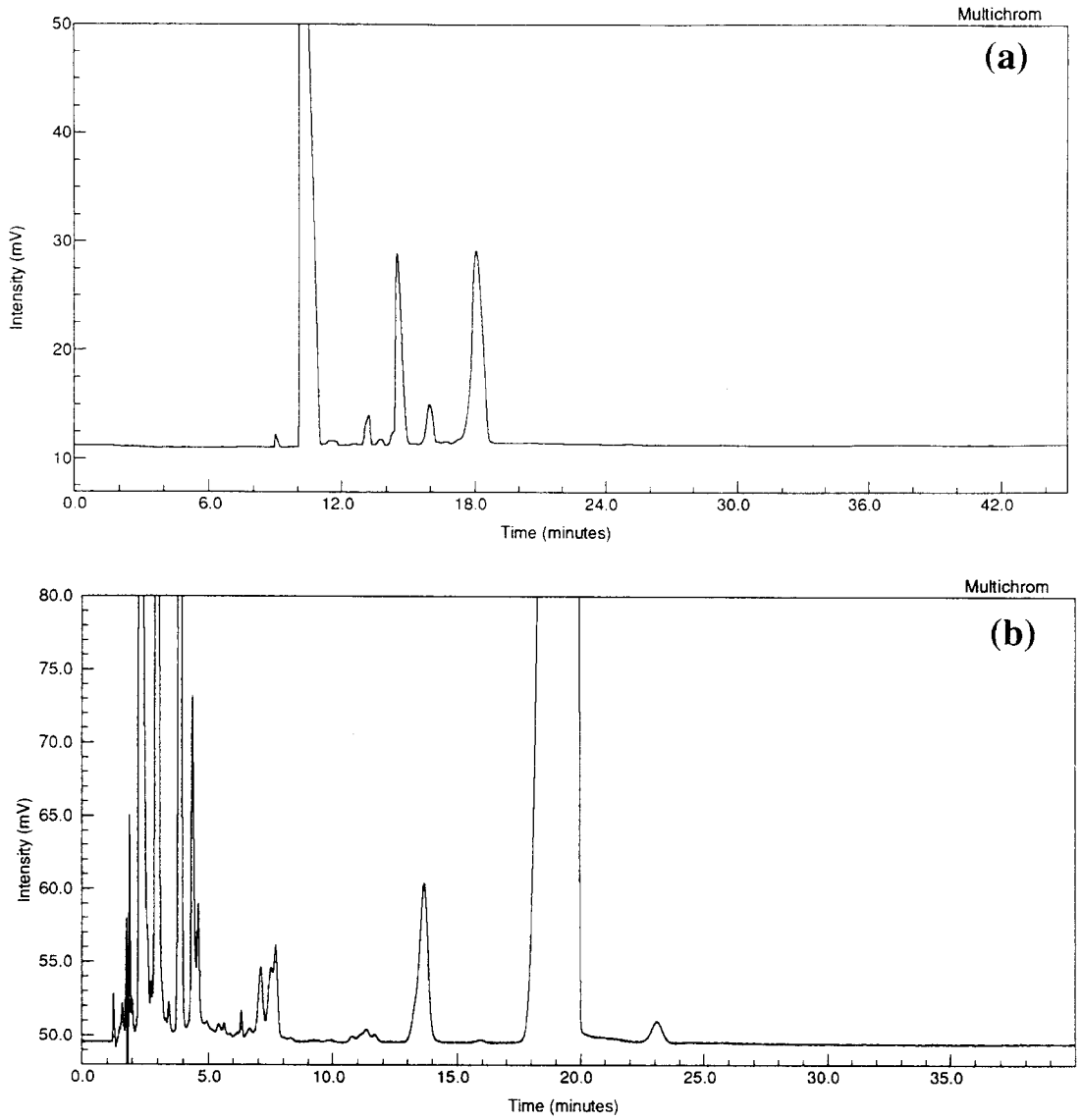

Fig. 7. CE Electropherogram (a) and HPLC chromatogram (b) of sample stressed under basic conditions

3. Results and discussion

3.1. CE assay validation

In order to confirm its suitability for use in the specificity studies, recovery experiments were performed using the CE assay. Triplicate samples representing 0, 80, 100 and 120% of the nominal mitoguazone dihydrochloride concentration were analysed. The results obtained are given in Table 1 which, for comparison, also gives data from the equivalent HPLC experiment. Although the average accuracy of the CE assay was satisfactory, its precision was not as good as that of the HPLC method. The coefficient of variation for the nine recovery results obtained by CE was 2.5% compared to that of 0.5% obtained by HPLC. It is possible that this is a function of the small injec-

tion volumes used (approximately 28 nl) in the former and that use of an internal standard would have improved its precision [8]. Given that, on this occasion, the CE assay was not developed for routine use, but part of a discrete validation exercise, further improvements in the assay precision were not sought. However, to partially compensate for the poorer precision, each sample was assayed in triplicate by CE for later comparison with the duplicate HPLC results.

3.2. CE sensitivity

A further consequence of the small injection volumes typically employed for CE is reduced concentration limit of detection. This is not generally an issue for drug substance assay methods, where the analyte injection strength can be readily adjusted, but it may become an important consideration in the detection of low impurity levels. Where sensitivity is the only consideration, this effect can be counteracted by use of lower wavelengths (down to 190 nm) to improve impurity responses but, since this work involved the direct comparison of responses obtained for species with varying UV/visible absorption spectra, it was important that the wavelength used for the HPLC determination (210 nm) was retained for the CE experiment. This being the case, dilute mitoguazone dihydrochloride standards, representing 0.1, 0.25, 0.5 and 1.0% of the nominal stressed sample content, were injected to investigate the method's sensitivity at 210 nm. The electropherogram obtained for the 0.1% w/w standard is given in Fig. 1 and confirms that the CE method had a sensitivity consistent with the detection of low levels of chromatographic impurities, at least down to this level, a pharmaceutically significant amount for impurities in drug substances [9].

3.3. Analysis of stressed samples

Stressed samples (see Section 2) were assayed by CE and HPLC whilst impurity levels were determined by CE, HPLC and TLC. Additionally, the homogeneity of the mitoguazone dihydrochloride peak obtained in the HPLC assay was assessed using data from a diode array detector. The data are summarised in Table 2 and correlation between the CE and LC results shown graphically in Figs. 2 and 3. Representative data from the diode array detector are presented in Figs. 4 and 5.

Agreement between the CE and HPLC assay data was excellent, providing together with the favourable mass balance, strong evidence that the HPLC assay was specific for mitoguazone dihydrochloride. The agreement between the CE and HPLC impurity data was also good. Where the stressed sample had an impurity level in excess of 2% by HPLC, the corresponding CE result was slightly lower (2–4%). Although the specific reason for this effect was not determined, it is possibly a function of using total sample area to calculate the CE impurity results versus the use of a dilute external standard to calculate those in the corresponding HPLC experiments. Alternatively, subtle differences in the sample environment experienced during CE and HPLC analysis may have lead to variations in impurity response. Notwithstanding this, these data support the view that there were no significant impurities which remained undetected using the HPLC methods.

The expected difference in selectivity between CE and HPLC is adequately demonstrated by examination of the various impurity elution profiles. Figs. 6 and 7 give these for the samples stressed under acidic and basic conditions respectively showing that the major species of interest are detected after the mitoguazone dihydrochloride peak using the CE method whilst the opposite is true for the HPLC method. On average, twice as many significant ($> 0.1\%$, w/w) impurity peaks were detected by HPLC than were detected by CE for samples which had impurity levels of at least 4% w/w. This reflects the fact that the CE conditions were not optimised for separation of the relevant impurities. Further optimisation could have been achieved, for example, by modification of the run buffer pH or by the use of additives such as cyclodextrins or surfactants. However, such modification proved unnecessary within the context of this study.

It is worth noting that agreement between the impurity results obtained by CE/HPLC and those obtained by TLC (Table 2) was also satisfactory,

even though the latter were based on visual estimation. Although, on this occasion, they provide good supporting evidence for the specificity of the HPLC systems being validated, the 'semi-quantitative' nature of this type of data is potentially a major disadvantage with respect to the use of a truly quantitative comparator such as CE, especially if the visualisation technique used elicits significantly different responses from the impurities with respect to the parent compound. However, TLC has at least one advantage over CE and HPLC in that the entire chromatogram is available for inspection so that species varying substantially in, for example, charge or polarity and which may be retained during CE or HPLC analysis are still readily detectable. Because of this, TLC is likely to continue to have a place as part of chromatographic specificity testing strategies.

Despite the choice of reverse phase HPLC for the routine analysis of mitoguazone dihydrochloride, its highly polar nature does not make it an ideal candidate for such a technique, hence the necessity of using an ion-pair reagent in the mobile phase. Given the agreement achieved between the HPLC and CE results, the data presented can equally be viewed as demonstrating that, with appropriate improvements in precision and following suitable additional validation experiments, the CE assay would be suitable for the routine determination of mitoguazone dihydrochloride drug substance.

4. Conclusions

CE has been successfully used to generate validation data in support of the HPLC assay and chromatographic impurity methods for mitoguazone dihydrochloride, providing strong evidence for the specificity of these methods. Not only does this serve as a further example of the growing importance of CE as a complimentary technique to HPLC for the analysis of pharmaceuticals but also suggests that CE could be used instead of HPLC for the routine assay of mitoguazone dihydrochloride.

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