# Determination of *N,N*-dimethylaminoethyl chloride and the dimethylaziridinium ion at sub-ppm levels in diltiazem hydrochloride by LC-MS with electrospray ionisation

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An LC–MS method is described for the determination of the synthetic reagent *N,N*-dimethylaminoethyl chloride (DMC) in the drug substance diltiazem hydrochloride, for which the permissible limit is not more than 1 ppm ($\mu$g g$^{-1}$). The *N,N*-dimethylaziridinium ion (DMA), the reactive intermediate formed by cyclisation of DMC, is also detected. A column switching arrangement is used: diltiazem hydrochloride is trapped on a reversed-phase HPLC column, and the polar analytes are separated by ion exchange chromatography. Ionisation is effected by positive-ion electrospray, and the quadrupole filter mass spectrometer is operated in the selected ion recording mode. The detection limit (peak height-to-baseline noise ratio = 3) for DMC varies from day to day in the range < 0.05 to 0.1 ppm. The response for DMC is linear ($r > 0.999$) over the concentration range 0.2–10 ppm, and the repeatability is better than 7% (relative standard deviation) at 1.0 ppm. Concentrations of DMC in diltiazem hydrochloride from the manufacturing facility under study ranged from undetectable to about 0.07 ppm. An indirect TLC method has been published for the determination of DMC in mepyramine maleate, but it lacks the necessary sensitivity and specificity. The LC-MS method presented is direct, straightforward and suitable for routine use.

## Introduction

The calcium antagonist diltiazem has been marketed for the treatment of cardiovascular disorders for more than 20 years, and it remains one of the leading products in this therapeutic area. The drug substance (Fig. 1) was originally developed as the hydrochloride, but the malate and maleate salts have also been described.

Diltiazem has, like many other drug substances, a (β-dialkylamino)ethyl side-chain. The synthesis of such compounds usually involves the alkylation under basic conditions of a nucleophilic intermediate with the corresponding tertiary β-chloroethylamine. In the case of diltiazem, the alkylating agent used is 2-chloro-*N,N*-dimethylethanamine hydrochloride, for which the commonly used trivial name is *N,N*-dimethylaminoethyl chloride hydrochloride (DMC). Limits for residual alkylating agents in drug substances are set many orders of magnitude below any level at which a toxic effect would be experimentally detectable, but without imposing an impossible challenge to the analyst. The authors' company applies a limit of not more than 1 ppm ($\mu$g g$^{-1}$) of DMC in diltiazem hydrochloride, and this is likely to be adopted by the regulatory authorities.
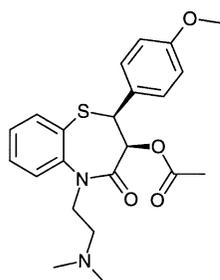
Only one method for the determination of this type of alkylating agent appears to have been published.[1] The compounds are hydrolysed to the corresponding alkanolamines, which are determined by TLC after derivatisation. A detection limit of 1 ppm was obtained for one of the compounds investigated, by use of a fluorescent derivative. The proposed limit requires a detection limit lower than this, and the utility of the method is limited by the fact that innocuous traces of alkanolamine present in the drug substance would be counted as alkylating agent.

Before discussing alternative analytical methods, it is necessary to review the chemistry of tertiary β-chloroethylamines, a subject that has been extensively studied because these compounds are closely related to the nitrogen mustards, RN($C_2H_4Cl$)$_2$.[2,3] The hydrochloride salts are almost unreactive as alkylating agents, and they are stable at room temperature in aqueous solutions of sufficiently low pH. Cyclisation to the corresponding aziridinium ions occurs in solution, as shown in Fig. 2 for DMC, at rates that are proportional to the non-protonated fraction.[3] The dimethylaziridinium ion (DMA) that is formed from DMC reacts only slowly with water, but such ions react relatively rapidly with other nucleophiles, including any buffer or salt anions that may be present.[4] Since reaction with chloride ion restores the original open-chain compound, the reaction is reversible when chloride is the dominant anion. As is to be expected, the equilibrium favours the neutral open-
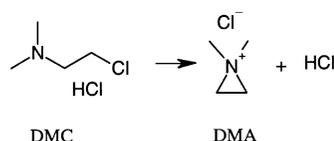


**Fig. 1** Structure of diltiazem.



**Fig. 2** Cyclisation of DMC to DMA and reaction of DMA with nucleophile.

chain form in non-polar solvents and during gas chromatographic analysis, and the aziridinium ion in aqueous solution.

In early studies, later confirmed by NMR,[5] the cyclisation reaction was monitored by determination of the chloride ion released. The concentration of aziridinium ion was determined by means of its reaction with thiosulfate (a soft nucleophile), and its hydrolysis was monitored by titration of the hydrogen ion released. A kinetic study of DMC in aqueous solution was reported in 1949.[6] The free base (p$K_a$ 8.4, estimated from the value of 8.82 for the N,N-diethyl homologue;[3] a published value[7] of 5.83 is incorrect) was obtained by adding 1 equiv. of NaOH to solutions of the hydrochloride. Conversion to DMA was practically complete within 2 h at 25 °C. The cyclised product reacts slowly with water, but the rate of formation of N,N-dimethylaminoethanol was increased in the presence of an excess of NaOH. As had been reported previously for related compounds,[3] the activation energy of the cyclisation is high, and aqueous solutions of DMC base were found to be stable for several hours at 0 °C (unpublished data from this laboratory).

It is clear that the reactive intermediate in the synthesis of diltiazem is DMA. Although it is most likely that any residue would be destroyed during subsequent steps in the process, the absence of DMA in the finished product must be proved. DMA could be re-converted into DMC in the case of diltiazem hydrochloride. Otherwise, it could be solvolysed, or converted into the O-acetate of N,N-dimethylaminoethanol (or to the corresponding esters in the case of the malate or maleate salts). An excess of DMC is likely to be used in any proprietary process, and therefore some of this starting material could be present in the reaction product, the amount depending on (among other factors) the polarity of the solvents used. Since DMC is stable in non-polar solvents and in neutral or acidic aqueous solutions, traces of residual reagent could appear in the finished product.

A GC method (unpublished) has been developed for DMC. Diltiazem hydrochloride is extracted as an ion pair with chloroform from an acidified aqueous solution, and then DMC is extracted into diisopropyl ether after addition of base. The flame ionisation detector is not sufficiently specific unless purified solvents are used, and a nitrogen-specific or MS detector would probably be required for routine use. The sum of DMC and DMA concentrations would be obtained by warming the aqueous extract after addition of HCl.

An unpublished method that has been used routinely determines the sum of the DMC and DMA concentrations by means of the general spectrophotometric reagent for alkylating agents 4-(4-dimethylamino)pyridine (NBP) (Fig. 3).[8,9] Alkylation of this reagent yields a colourless pyridinium ion, which on addition of a strong base is converted into the violet tetra-
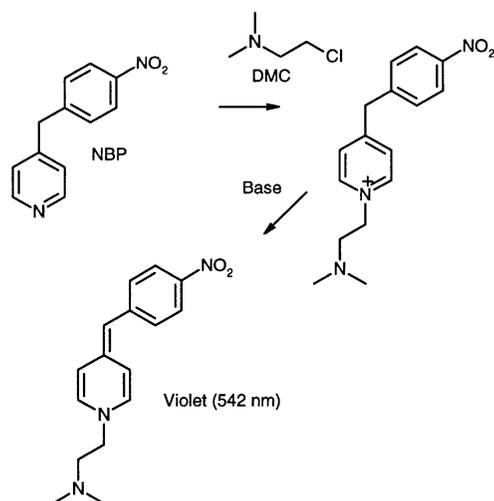
hydropyridine. Blank values, about 0.5–0.8 ppm, are high but acceptable for the present purposes, since the actual concentrations in production batches (determined by LC-MS) are invariably less than 0.1 ppm. Any interference by other alkylating agents would not be considered a disadvantage in the present case. High blank values and also instability of the coloured reaction product are commonly reported in the literature, and it is usually necessary to recrystallise the commercial reagent. Recently, it has been shown that the stability can be improved by the formation of an inclusion complex with a cyclodextrin.[10] In preliminary experiments, it was found that better specificity and a detection limit of the order of 0.1 ppm can be obtained by HPLC analysis of the pyridinium intermediate, the tetrahydropyridine (which is stable only in strongly alkaline solution) being generated by post-column addition of base. A few other reagents whose specificity depends on an alkylation-induced spectral shift have been proposed, but they do not appear to be in widespread use. The use of a specific detection method such as LC-MS would permit a wider choice of nucleophilic derivatising agents, an approach that was not pursued for the reasons given below.

All the above methods are fairly tedious and they require several extraction steps. Moreover, a sensitive reference method that differentiates between DMC and DMA is needed in order to follow the transformations that occur during the synthetic process. In this paper, we describe a direct LC-MS method with electrospray ionisation that has the required sensitivity and specificity. At first sight, this technique would seem unpromising for analytes of such low molecular mass, because of the presence of mobile phase impurities and ion clusters. However, a column-switching system allows the injection of amounts of drug substance sufficient to render the method robust. The equipment used is nowadays considered standard, and no sample preparation other than dissolution is required.

## Experimental

### LC-MS

Two identical LC-MS sets (Waters, Saint Quentin, France) were used. The chromatographs (Alliance 2690) were fitted with in-line vacuum de-gassers and operated isocratically. A diode-array UV absorbance detector (Model 996) was used during method development to monitor the elution of diltiazem. An auxiliary pump (Jasco BIP1, sold by Merck, Nogent-sur-Marne, France or Model 1050 Hewlett-Packard, Courtaboeuf, France) was used for column switching. The chromatograph was remotely controlled by means of the MS software. The motorised six-port injection/switching valve fitted to the MS was wired to external events contacts on the chromatograph, because direct control by the MS software has not yet been implemented.

Two HPLC columns were connected as shown in Fig. 4. The mobile phase supplied by the Waters pump consisted of 100 mM aqueous ammonium acetate adjusted to pH 2.9 with formic acid, and acetonitrile (4 + 1 v/v). The flow rate was 0.6 ml min$^{-1}$. Injections (20 µl) were carried out with the Alliance autosampler, with the switching valve in the position marked 'inject'. Diltiazem is retained by the first column (Purospher C$_{18}$, 125 × 4 mm id, 5 µm), while the unretained polar analytes are transferred to the cation exchange column (Hypersil SCX, 150 × 3 mm id, 5 µm). After 3 min, the valve is switched to the position marked 'load'; the reversed-phase column is purged with a mixture of acetonitrile and water adjusted to pH 2.9 with formic acid (3 + 1). The valve is returned to its original position at the end of the run (17 min), and the system is allowed to equilibrate for 5 min before the next injection.



**Fig. 3** Spectrophotometric determination of DMC + DMA.

Two quadrupole (Model LCZ) mass spectrometers with Masslynx version 3.1 software were used. They were operated in the positive-ion electrospray mode, with a 10:1 split at the mobile phase inlet. Typical operating conditions were as follows: capillary 3.9 kV, cone 32 V, extractor 3 V, entrance hexapole 0.3 V, source temperature 100 °C, desolvation temperature 150 °C, low mass resolution 13.0, high mass resolution 13.1, ion energy 0.5 V, photomultiplier 680 V and nitrogen flow rate 400 l h$^{-1}$. Selected ion recording was carried out at $m/z$ 108 (DMC, protonated molecule) and 72 (DMA, quaternary ammonium ion), with a dwell time of 1 s per ion, an inter-channel delay of 0.02 s and a mass span of 0.3 u.

## Sample preparation

Stock standard solutions of DMC (1 mg ml$^{-1}$) were prepared in the injection solvent, a mixture of acetonitrile and water adjusted to pH 2.5 with formic acid (1 + 4). Dilutions were carried out with the same solvent. Safety precautions were that: weighings and initial dissolution were carried out in a laminar-flow safety cabinet adapted for the use of a microbalance, and DMC was destroyed by overnight reaction in an aqueous solution containing 5% each of sodium bicarbonate and sodium thiosulfate.

DMA was prepared by dissolving about 16 mg of DMC (accurately weighed) in water in a 10 ml calibrated flask. After addition of 1.5 ml of 0.1 M aqueous KOH, the solution was made up to the mark with water and the flask was maintained at 25 °C for 3 h. LC-MS analysis of 20 000-fold dilutions showed that cyclisation (Fig. 2) is complete within 2 h, with formation of no more than traces of N,N-dimethylethanolamine, in accord with the published data. For the purposes of this study, it is assumed that the yield of DMA is stoichiometric.

Diltiazem hydrochloride and intermediates were dissolved in the injection solvent (50 mg ml$^{-1}$; 1 mg injected).

## Results and discussion

### Operating conditions

Ion exchange chromatography was used, together with a largely aqueous mobile phase, since the analytes are highly polar and the drug substance is readily soluble in water. DMC is stable in water when fully protonated (pH less than about 4). Reversed-phase ion-pairing chromatography was not considered, as only a limited number of ion-pairing agents are compatible with atmospheric pressure ionisation. A conventional high-capacity silica-based cation exchange column was used, as it is inexpensive and robust; no change in retention times beyond normal day-to-day variations (Fig. 5) was observed for more than 700 injections. Low-capacity stationary phases of the type used for ion chromatography were not investigated; they would have allowed the use of a less concentrated buffer solution as mobile phase, but we have experienced no difficulty in the use of relatively high concentrations of ammonium formate and acetate, even for determinations at low $m/z$ values. Several hundred injections have been performed, during which time the only maintenance required was occasional cleaning of the entrance cone, which takes only a few minutes.

Startin et al.[11] have discussed the use of ion exchange chromatography with electrospray ionisation for the determination of residues of the quaternary ammonium ion chlormequat in pears. The best long-term stability was obtained with an ion source which, like that used for the present work, uses orthogonal ion extraction. A source of older design was found to be unusable, but this problem may have been due in part to the absence of clean-up of the biological extracts. These authors noted a rapid deterioration of their ion exchange column, which may have been due to contamination, or to the use of a mobile phase (ammonium formate without pH adjustment) of higher pH than ours. Stationary phase leaching is a frequent source of baseline noise and contamination in LC-MS; our results show that the ion exchange column used is stable, at least at pH 2.9.

Sensitivity was found to be better with electrospray ionisation than with atmospheric pressure chemical ionisation (APCI). Conditions were optimised in order to minimise fragmentation of diltiazem, which yields a fragment ion having the molecular formula of DMA ($m/z$ 72). Under these conditions, the baseline noise at $m/z$ 72 and 108 was such that injections of the order of 1 mg of diltiazem hydrochloride were necessary in order to obtain adequate margins for the signal-to-noise ratios at the target concentration of 1 ppm of DMC or DMA. Diltiazem has a longer retention time than the analytes on the ion exchange column, but the large amount injected could not be completely purged from the column within a reasonable time. This problem was solved by trapping the drug substance on a reversed-phase column, on which the analytes are unretained; this column is efficiently purged by a mobile phase containing a higher percentage of organic solvent. The capacity of the reversed-phase column was not evaluated. If necessary, larger injections
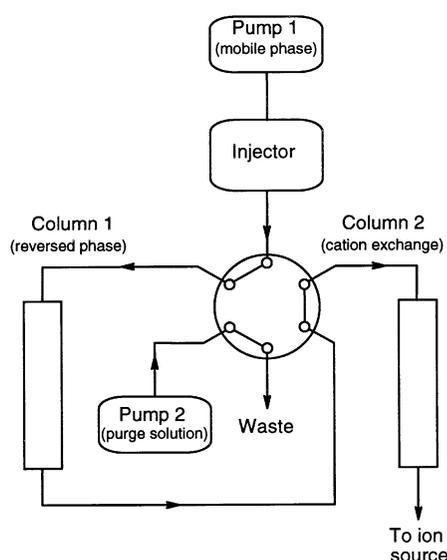


**Fig. 4** Column switching system. The valve is shown in the position marked 'Load'.
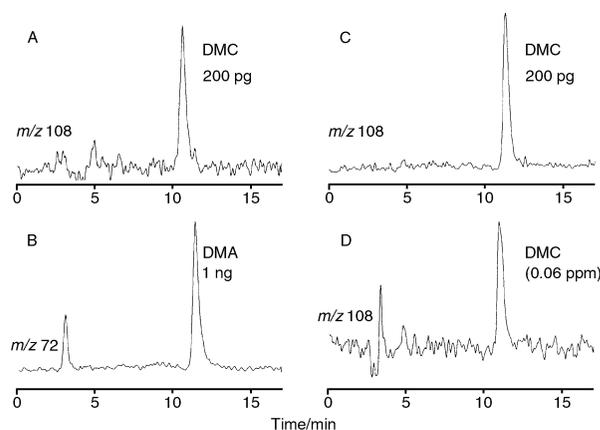


**Fig. 5** Chromatograms (selected ion monitoring): (A) DMC (200 pg, corresponding to 0.2 ppm), operator 1; (B) DMA (1 ng, corresponding to 1 ppm), operator 1; (C) DMC (200 pg), operator 2; (D) batch of diltiazem hydrochloride estimated to contain 0.06 ppm DMC (operator 1). Normal day-to-day variation in retention times is illustrated; DMA elutes later than DMC ($R_s$ = 1.6).

could be accommodated by increasing the size of this column and using gradient elution for the ion exchange separation.

No build-up of contamination has been observed that would interfere with the analysis. On the other hand, the injector showed a memory effect for diltiazem that proved troublesome during subsequent unrelated studies. The model of injector used is not the most appropriate for such large injections; it was chosen for its other qualities,[12] and a different type (or a dedicated injector) will be used for future analyses. Apart from this problem, the method has proved in practice more convenient and straightforward than any of the alternatives mentioned in the Introduction.

Representative chromatograms are presented in Fig. 5. DMC and DMA are separated from each other ($R_s$ = 1.6) on the ion exchange column, although a separation is not strictly required as the mass spectra are different. The target analytes are well separated from *N,N*-dimethylaminoethanol (typical retention time 6.9 min) and its *O*-acetate derivative (9.8 min). These two compounds are detectable by their protonated molecular ions or by a fragment ion of *m/z* 72. Since the concentrations were undetectable in diltiazem hydrochloride from the authors' company, and in the low ppm range in second crops and in diltiazem from other sources, these trace impurities were not studied further.

## Validation

Validation data were obtained by two operators working independently with different columns on different LC-MS sets (which were, however of the same make and model). Guidelines for validations in pharmaceutical analysis have been issued by the International Committee on Harmonisation (ICH), and a protocol[13] associated with a commercially available software package[14] has been published. In view of the technique used and the sensitivity required, it was not possible to follow this protocol rigorously; parameters such as baseline noise and instrumental response would vary significantly during the long sequences of injections required. In order to take into account the degree of variability encountered in practice (*e.g.*, with respect to the state of the ion source and ion optics and trace impurities in the mobile phase), the discussion below takes into account information obtained during routine application of the method, in addition to data from the formal validation exercise.

Detection limits (ratio of peak height to peak–peak noise over 20 peak half-widths = 3) were evaluated after digital smoothing. The smoothing, which had negligible effects on peak height and width, resulted in a threefold improvement in signal-to-noise ratio. Operator 1 consistently obtained detection limits of 0.05 ppm or lower for DMC (Fig. 5C). Detection limits obtained by operator two were generally higher than than this, and ranged from 0.05 to 0.1 ppm during the study (about 0.1 in Fig. 5A).

For operator 1, the precision (RSD) for repeat injections, determined on several occasions, was almost always less than about 2% throughout the concentration range 0.2–10 ppm. The only exception to this was a value of 6.6% at 0.3 ppm, obtained during validation of the choice of quantification limit (Table 1). The corresponding value obtained by operator 2 was 2.9%; on this occasion, both operators obtained detection limits of about 0.05 ppm. Analysis of variance of the data from six independent calibration curves prepared by operator 2 gave RSD values for repeat injections of 10, 6.2, 3.5, 1.6 and 1.9% at 0.2, 0.5, 1.0, 2.0 and 5.0 ppm, respectively. This trend is consistent with the presence of a significant amount of baseline noise, and in fact the detection limit was 0.1 ppm on this occasion, the highest encountered during the project. Note, however, that the detection limit according to the above definition is a poor

indicator of the precision in absolute terms, as its value can be varied by a suitable choice of smoothing algorithm.[15] On different occasions, RSD values obtained by operator 2 ranged from 1.8 to 4.4% at 1 ppm. For reference, with UV absorbance detection, the HPLC equipment used gives RSD values of less than 0.3% for the areas of peaks of adequate intensity.

In view of the above results, linear regression analyses would be influenced if not invalidated by errors in making up the solutions and by instrumental drift, which amounted to about 10% per 24 h. Instrumental linearity was evaluated in the presence and absence of diltiazem hydrochloride taken from a batch containing no detectable DMC, for six concentrations of DMC (0.2, 0.5, 1, 2, 5 and 10 ppm). A single stock standard solution was diluted 100-fold and then serially diluted for each curve. Operator 1 prepared one pair of curves, injecting each solution three times. Operator 2 prepared three independent pairs of curves, injecting each solution twice. The order of the solutions was randomised within each calibration curve, but repeat injections were performed consecutively. Visual inspection revealed no evidence for deviation from linearity. Subsequently, however, data for 10 ppm were rejected for statistical analysis, because the variance was not homogeneous and the concentrations found were all well below this value. Unweighted linear regression analysis was carried out, because the precision as a function of concentration was not the same for the two operators: for operator 1, the mean RSD for repeat injections was 1.8% and independent of concentration, whereas the values obtained by operator 2 varied as described above. For all eight curves, the confidence limits for the intercepts included the origin, and the regression coefficients ranged from 0.9996 to 0.9999. The test for lack of fit indicated no significant deviation from linearity. The standard deviation of peak surface area on concentration ($S_{y/x}$), expressed as a percentage of the area at the centroid of the curves (corresponding to a concentration of 1.7 ppm), ranged from 1.3 to 3.3%.

The within-day repeatability and recovery were evaluated by both operators for 1 ppm of DMC. Six independent solutions contained DMC only and six contained diltiazem hydrochloride spiked with DMC. The relative standard deviations (Table 1) are, as expected, higher than would be indicated by the regression analysis above. However, all values are well within the norm of 10% that is currently applied to impurities at concentrations of the order of 1000 ppm (limits for the precision of trace impurity determinations are not specified by the guidelines). The recovery of DMC from diltiazem hydrochloride (Table 1) was estimated from the two repeatability experiments, and also from the linearity data obtained by operator 2 (the linearity data obtained by operator 1 were not used for this purpose because of instrumental drift). None of the values obtained was significantly different from 100%.

The detection limit for DMA was similar to that for DMC (0.15 ppm for the chromatogram shown in Fig. 5B). A full validation for DMA was not carried out in view of the negative results of batch analyses (below), but the chromatograms

**Table 1** Repeatability and recovery data for DMC (*n* = 6)

| | Relative standard deviation (%) | | | |
| | | | | |
| Operator | Repeatability, DMC alone (1 ppm) | Repeatability, diltiazem hydro-chloride + DMC (1 ppm) | Injection repeatability, DMC (0.3 ppm) | Recovery (%) |
|---|---|---|---|---|
| 1 | 4.2 | 5.6 | 6.6 | 97.3 |
| 2 | 4.7 | 5.1 | 2.9 | 98.7 |
| 2 | | | | 104[a] |
| | | | | 94[a] |
| | | | | 102[a] |

[a] Ratio of slopes of three independent pairs of calibration curves.

obtained justify the use of the method as a limit test for this compound with a limit of 1 ppm.

The injection solutions are stable for at least 38 h (98% recovery with respect to freshly prepared solutions).

## Application of the method

To date, 31 production batches of diltiazem hydrochloride from the authors' company have been examined. Two batches gave no discernible peak for DMC, and estimated concentrations in the other batches (at or below the detection limit) ranged from 0.02 to 0.06 ppm. A chromatogram of a sample estimated to contain 0.06 ppm is presented in Fig. 5(D). Seven samples of diltiazem hydrochloride obtained from three other manufacturers were examined. Concentrations ranged from undetectable to 0.7 ppm. These results demonstrate that the formation of an artefact during the analysis of diltiazem hydrochloride by a pharmacopoeial method for volatile impurities is not due to the presence of DMC.[16]

No sample of the drug substance gave a discernible peak for DMA. Three batches of the product obtained by work-up of the alkylating reaction mixture were analysed; one of these showed a small peak at the retention time of DMA, corresponding to <0.1 ppm. Levels of DMC at this stage of the process are in the range 1–4 ppm. These results suggest that the DMC in the finished product arises practically exclusively from unreacted DMC remaining after the alkylation reaction, and not by reversal of the cyclisation reaction. It must be emphasised that this conclusion applies only to the industrial process that was studied.

The data presented demonstrate that the detection limits, precision, accuracy and specificity of the method are adequate for monitoring DMC and DMA in diltiazem hydrochloride at the proposed limit of 1 ppm. Day-to-day variations in performance criteria remain within acceptable limits. However, the nature of the technique is such that, in the absence of a co-eluting isotopically labelled internal standard, the linearity of the response should be verified on each occasion the method is applied, particularly with different instruments. In addition, since with electrospray ionisation the response to many compounds is known to be influenced by low concentrations of co-eluting substances, the recovery should be evaluted for each source of diltiazem hydrochloride examined. It is recommended, therefore, that quantitative analyses be carried out with reference to a calibration curve, and that recovery data be obtained on each occasion the method is used.

The method does not provide positive identification of the peaks that were detected at the appropriate retention times; drug substances may contain other impurities at the ppm level, and fragment ions may interfere with the detection of the analytes at low $m/z$ values. Determination of the isotope ratio of the molecular ion cluster ($m/z$ 108/110) of DMC would provide some additional assurance, but electrospray ionisation does not supply the same quality of evidence as electron ionization. A spectrum could not be obtained at the 1 ppm level during preliminary experiments by GC–MS (EI/CI source and quadrupole filter), but further preconcentration of the sample or use of a more sensitive type of mass spectrometer could be envisaged if proof of identity is required. Possible alternative confirmatory methods are tandem MS and medium- or high-resolution MS. The latter greatly increases the specificity for small molecules,[17] but at present the technique is routine only with magnetic sector instruments, which are not usually available in pharmaceutical analysis laboratories. While current time-of-flight mass spectrometers have the necessary resolution (3000–5000), the apparent $m/z$ ratio at the peak centroid varies with concentration, as a result of detector dead time. Quadrupole ion trap mass spectrometers also show apparent $m/z$ shifts, although for different reasons.[18]

Finally, it was not established whether the counter-anion of diltiazem hydrochloride is retained as an ion pair by the reversed-phase column, or whether it passes unretained into the ion source as ammonium chloride. In any case, no difficulty was experienced during long series of injections. Other salts of diltiazem may require the installation of a diversion valve between the ion exchange column and the ion source.

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