ELSEVIER

Journal of Chromatography B, 708 (1998) 113–120

JOURNAL OF CHROMATOGRAPHY B

Validated method for the determination of idazoxan in human plasma by liquid chromatography with tandem mass spectrometric detection

N.C. van de Merbel[a,*], A.P. Tinke[1,a], W.D. van Dongen[a], B. Oosterhuis[a], J.H.G. Jonkman[a], Ph. Ladure[b], C. Puozzo[b]

[a]*Pharma Bio-Research International, P.O. Box 200, 9470 AE Zuidlaren, Netherlands*
[b]*Institut de Recherche Pierre Fabre, Rue Jean Rostand BP 687, 31319 Labege Innopole Cedex, France*

Received 8 September 1997; received in revised form 8 December 1997; accepted 16 December 1997

Abstract

A liquid chromatography–tandem mass spectrometry method for the determination of idazoxan in human (heparin) plasma is presented, which was developed and validated using 500 μl of sample. Sample preparation consisted of the addition of fluoroidazoxan as the internal standard, extraction at alkaline conditions into *tert*.-butyl methyl ether, followed by centrifugation, evaporation of the solvent and reconstitution in methanol. After a short chromatographic run, detection took place by ionspray tandem mass spectrometry in positive ion mode. Validation results on linearity, specificity, accuracy, precision and stability, as well as application of the method to samples from a clinical trial, are shown. The validated calibration range is from 0.300 to 100 ng/ml, with accuracy (bias) and precision (coefficient of variation) being below 15% at all levels. A sample throughput of, typically, 150 per day can be achieved. © 1998 Elsevier Science B.V.

Keywords: Idazoxan

1. Introduction

Idazoxan is a potent and highly selective α_2-adrenoceptor antagonist that enhances the release of noradrenaline and modulates dopamine levels in the brain. It has been investigated in clinical trials for the treatment of depression and is presently being evaluated as a treatment for neurodegenerative disorders that are characterized by decreased catecholamine levels, such as Parkinson's disease.

For the determination of plasma levels after administration of idazoxan, initial studies employed column liquid chromatography (LC) with low-wavelength UV detection [1]. Unfortunately, this technique has a rather limited sensitivity, allowing the quantitation of idazoxan in plasma down to approximately 10 ng/ml, whereas, for the evaluation of pharmacokinetic parameters after low-dose studies in humans, an assay with a sensitivity of preferably 0.5 ng/ml would be required. Therefore, over the last decade, several other analytical methods were developed for the determination of idazoxan in plasma at the low and sub-ng/ml level.

Two binding assays, a radioreceptor assay [2] and

*Corresponding author.
[1]Present address: Yamanouchi Europe, Research Laboratories, Elisabethhof 1, 2353 EW Leiderdorp, Netherlands.

0378-4347/98/$19.00
PII S0378-4347(97)00672-5

a radioimmunoassay (RIA) [3] were described. The former is based on incubation of plasma samples with brain tissue homogenate (containing idazoxan-specific receptors) and ^{3}H-labelled idazoxan, the latter on incubation with idazoxan-specific antibodies and ^{125}I-labelled idazoxan. Although the sensitivity was improved compared to the LC method, viz. to 1 ng/ml for the radioreceptor assay and to 3 ng/ml for the radioimmunoassay, both methods have the disadvantage of being quite laborious and having a rather limited linear range of typically only one order of magnitude. A gas chromatographic method using a short non-polar bonded-phase capillary column and mass-selective detection allowed idazoxan to be determined without prior derivatization at sub-ng/ml levels and up to 50 ng/ml, but here also, the sample preparation procedure was quite time-consuming and a throughput of only 30 samples per day was achieved [4].

In order to further improve the sensitivity and sample throughput of the determination of idazoxan, the use of LC with tandem mass spectrometric (MS–MS) detection seemed a logical choice. Over the past few years, this technique has gone through tremendous developments and is now well-established for the routine determination of a variety of compounds, where it frequently combines a better sensitivity and a shorter run time compared to conventional methods. In this paper, the development and validation of a straightforward and rapid LC–MS–MS method for the determination of idazoxan in human plasma, using a simple liquid extraction step for sample preparation is described, and the method is compared to other available methods. To illustrate its applicability, the use of the method for clinical study samples is presented.

2. Experimental

2.1. Chemicals

Idazoxan hydrochloride was obtained from Sigma (St. Louis, MO, USA) and fluoroidazoxan hydrochloride (the internal standard) was provided by Institut de Recherche Pierre Fabre (Labege Innopole Cedex, France). Ammonium acetate and disodium tetraborate decahydrate were purchased from Merck (Darmstadt, Germany) and methanol, acetonitrile and *tert.*-butyl methyl ether from J.T. Baker (Deventer, Netherlands). Water was purified using a Milli-Ro-15 or a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

A Waters (Milford, MA, USA) Model 717 plus autosampler was used to introduce 15 μl aliquots of the pretreated samples into the chromatographic system. Reversed-phase liquid chromatography was performed using a 3-μm YMC-TMS column (50× 4.0 mm I.D.), obtained from YMC (Wilmington, NC, USA), which was conditioned at 25°C in a WO electronics (Langensdorf, Germany) Model BFO-04 column thermostat. A Perkin-Elmer (Beaconsfield, UK) Model 200 LC pump was used to deliver the eluent, a mixture of 0.01 *M* ammonium acetate (at native pH, which is approximately 6.9) and acetonitrile (10:90, v/v), at a flow-rate of 1.0 ml/min. The eluent was split in a ratio of 1:50 before it was introduced into the mass spectrometer.

Quantitation was performed by tandem mass spectrometric detection with a Perkin-Elmer Sciex API III plus mass spectrometer, using the ionspray inlet probe at 60°C. Detection of the ions was performed in multiple reaction monitoring (MRM) mode, monitoring the decay of the m/z 205 precursor ion to the m/z 97 product ion for idazoxan and of the m/z 223 precursor ion to the m/z 97 product ion for fluoroidazoxan in positive ion mode.

2.3. Standard solutions

A methanolic idazoxan stock solution (1000 μg/ml as idazoxan base) was used to prepare a series of working solutions containing idazoxan at concentrations of 10.0 μg/ml, 1000 ng/ml and 100 ng/ml, respectively, in water. These working solutions were used for the preparation of calibration and validation samples by adding of small volumes to blank human plasma. A stock solution of the internal standard, fluoroidazoxan (1000 μg/ml as the hydrochloride), was prepared in acetonitrile and was used to prepare a working solution (100 ng/ml).

Calibration samples were prepared with the following plasma concentrations: 0.500, 1.00, 5.00,

10.0, 25.0, 50.0, 75.0 and 100 ng/ml. Later, an additional calibration sample was used at 0.300 ng/ml. Validation samples were prepared with the following concentrations: 0.500, 1.00, 10.0 and 75.0 ng/ml and, later, also 0.300 ng/ml. Calibration and validation samples were prepared from separate stock and standard solutions.

2.4. Sample preparation

In the original method (0.500–100 ng/ml), an aliquot containing 1000 μl of (heparin) plasma was used, to which 100 μl of the internal standard working solution and 1000 μl of a 50 m*M* sodium borate buffer (pH 10.5) were added. After vortex-mixing for 5 s, 5.0 ml of *tert*.-butyl methyl ether was added, followed by extraction for 15 min using a tumble mixer at 40 rpm and centrifugation for 10 min at 3200 *g* at 4°C. The aqueous layer was discarded and the organic layer was evaporated to dryness under a gentle stream of nitrogen at 45°C. The residue was redissolved in 250 μl of methanol by vortex-mixing for 30 s, transferred into an autosampler vial and 15 μl was injected into the LC–MS–MS system.

An improved sample preparation procedure was employed later (0.300–100 ng/ml) using 500 μl of plasma. The other steps were similar to the first procedure, except that, after evaporation, the residue was redissolved in 100 μl of methanol.

2.5. Validation experiments

In the first instance, the method was validated over the range 0.500–100 ng/ml, using the validation approach as described in detail by Wieling et al. [5].

The linearity of the method was established by plotting the peak area ratio of idazoxan over internal standard versus its concentration (weighting factor, $1/x$) using eight calibration samples in triplicate and performing a goodness of fit and lack of fit test by analysis of variance (ANOVA) based on the following equation:

$$y = ax + b.$$

The specificity of the assay was checked by analysing blank human plasma samples obtained from different healthy subjects.

Accuracy and within-run and between-run precision were determined by ANOVA at the four levels mentioned above, by analysing samples in triplicate during five analytical runs.

The recovery was determined at three concentration levels (1.00, 10.0 and 75.0 ng/ml) by comparing the response of validation samples to the response of injections of standards at the same level performed in the same run. The recovery was calculated versus (i) aqueous standards and (ii) spiked extracts of blank plasma samples.

The stability of idazoxan was assessed at two concentration levels (1.00 and 75.0 ng/ml). Freeze–thaw stability in plasma was determined by repeatedly ($n=5$) freezing and thawing plasma samples and analysing the sample in triplicate after each freeze–thaw cycle in five analytical runs. The stability of idazoxan in the pretreated sample at 10°C, during storage in the autosampler, was determined by repeated injection of pooled extracts every hour for a period of 10 h. Benchtop stability of idazoxan in plasma at room temperature was established over a period of 24 h by taking samples at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h.

Later, the calibration range was extended to 0.300–100 ng/ml. A limited additional validation was performed; accuracy and within-run and between-run precision were established at 0.300 ng/ml by ANOVA after analysis in triplicate during two analytical runs.

3. Results and discussion

3.1. Method performance

Full scan mass spectra revealed that, for idazoxan (molecular mass 204), the ion at m/z 205 was formed to the largest extent (Fig. 1A), which obviously corresponds to the protonated molecule $[M+H]^+$. In addition, a number of less abundant species were formed, of which m/z 246 most probably represents a cluster of the protonated molecule with acetonitrile, which is the major constituent of the eluent. Under MS–MS conditions, applying a collision energy of 20 eV, the m/z 205 ion was found to

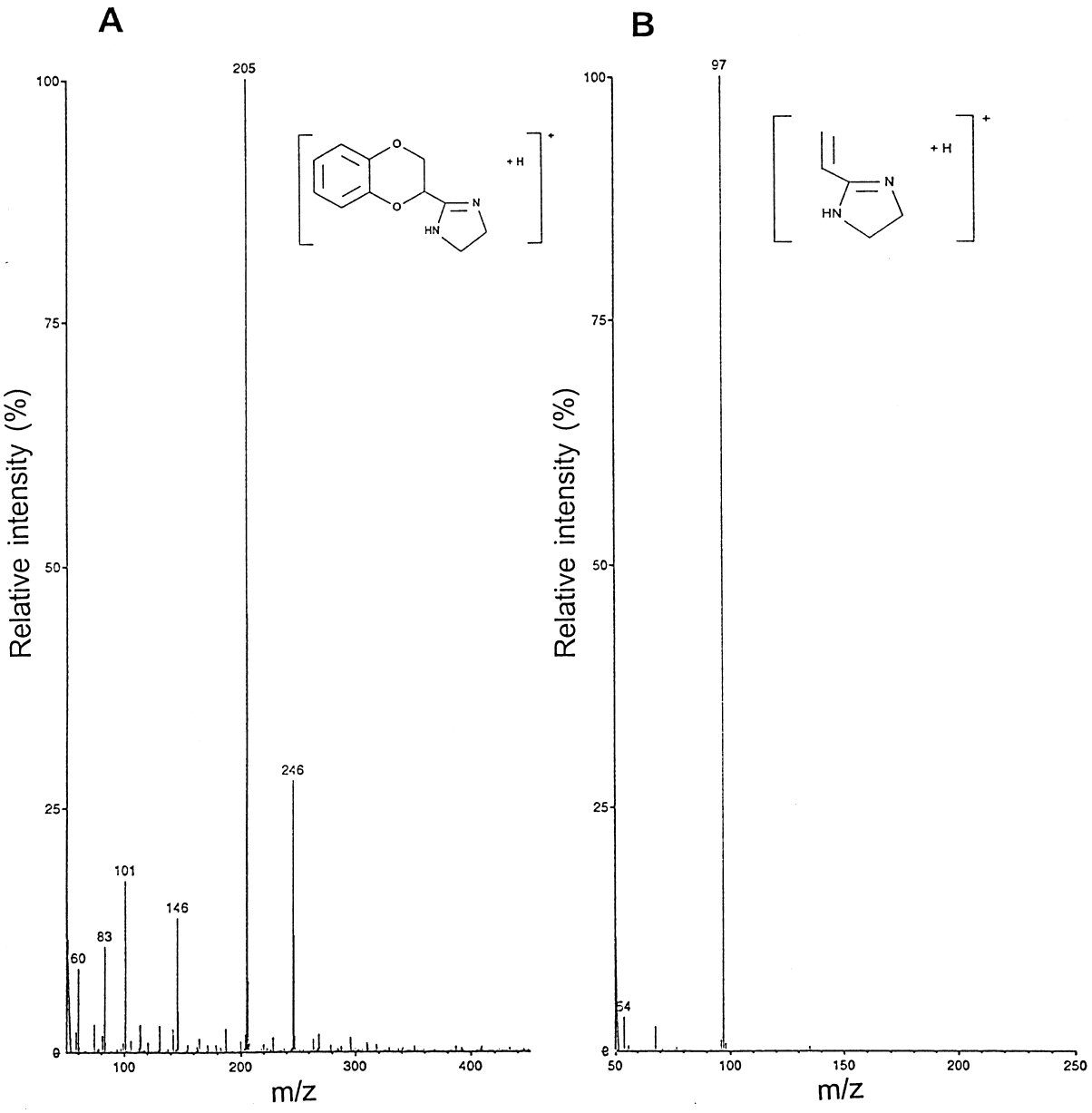

Fig. 1. Full scan (A) and daughter (B) mass spectra of idazoxan plus postulated structures.

fragment almost exclusively to a m/z 97 product ion (Fig. 1B). It could be speculated that the formation of this ion is caused by the cleavage of two C–O bonds in the 1,4-benzodioxane moiety, finally resulting in a protonated form of 2-vinylimidazoline, as represented in Fig. 1B. For the internal standard, fluoroidazoxan (molecular mass 222), a similar behaviour was observed: the most abundant ion formed was the protonated molecule with m/z 223, which fragmented predominantly to a m/z 97 product ion.

The detection of idazoxan using these conditions turned out to be highly selective and no interfering components whatsoever were observed in the chromatograms of plasma samples. Therefore, no full chromatographic separation was necessary and an analytical run time as short as just 2.30 min was found to be more than sufficient. This was accomplished by using a short (5 cm) LC column and a high modifier content (90% acetonitrile) in the eluent. To illustrate this, Fig. 2 shows chromatograms of blank plasma, plasma spiked at the lower limit of quantitation (0.300 ng/ml) and the internal standard at the concentration used (10.0 ng/ml).

Probably because of (i) the favourable ionization characteristics of idazoxan ($pK_a = 8.6$), which is completely in the protonated form in the column effluent (pH 6.9), (ii) an efficient desorption of the charged analyte from the solvent during the ionspray process and (iii) the favourable fragmentation of idazoxan to just a single product ion (see Fig. 1B), the detection as such was found to be highly sensitive. As a result, a rather simple and rapid sample preparation scheme was developed, consisting of the extraction of idazoxan from plasma at a high pH into an organic solvent, followed by evaporation of the solvent, reconstitution of the residue and injection into the analytical system. Starting with 500 μl of plasma and reconstituting into 100 μl of methanol, a fivefold enrichment was achieved, which, quite easily, enabled the quantitation of idazoxan down to 0.300 ng/ml using an injection volume of only 15 μl.

3.2. Validation results

The linearity of the method was established for the concentration range of 0.500–100 ng/ml during the original validation. Analysis of variance indicated that the linear model with a weighting of $1/x$ is appropriate for establishing a relationship between the concentration and the response. No significant lack of fit was observed and the goodness of fit was highly significant (data not shown). For this validation, as well as for that of the extended concentration range, correlation coefficients above 0.998 were found.

As already mentioned above, the selectivity of the method was very high and no interfering compounds were found in the plasma of different healthy subjects. The detection limit was determined by background noise rather than by interference of endogenous plasma components; it was estimated to be 0.1 ng/ml, using 500 μl of sample. For practical reasons, the lowest point of the calibration curve, 0.300 ng/ml, was taken as the lower limit of quantitation (LLQ). As can be concluded from Table 1, precision and accuracy at this level were well within the limits (bias and C.V. <20%) established for this purpose [6].

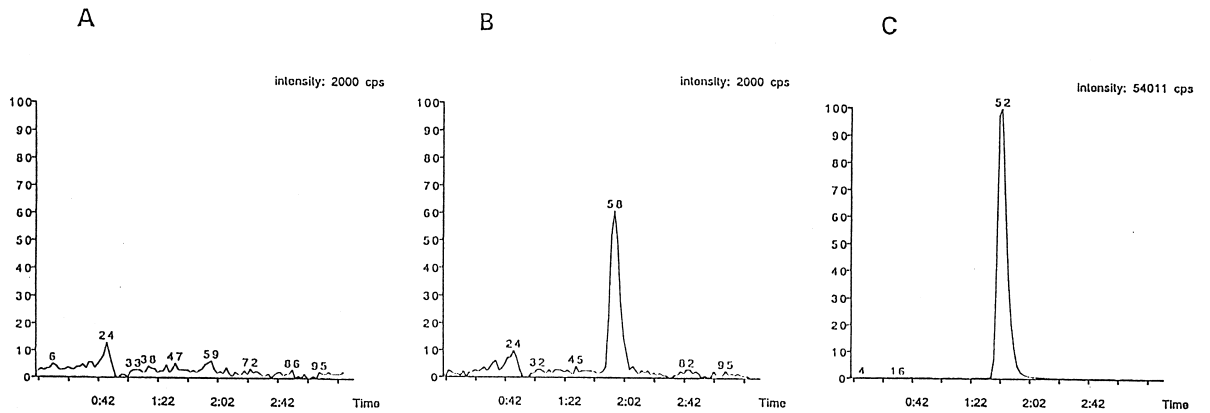

Fig. 2. LC–MS–MS chromatograms of blank human plasma (A) and human plasma containing 0.300 ng/ml idazoxan (B) and 10.0 ng/ml fluoroidazoxan, the internal standard (C).

Table 1
Summary of accuracy (expressed as percentage bias), precision (expressed as percentage C.V.) and recovery of idazoxan in plasma samples

Nominal concentration (ng/ml)	Bias (%)	Within-run C.V. (%)	Between-run C.V. (%)	Recovery versus aqueous standards (%)	Recovery versus spiked extracts (%)
0.300	+12.8	6.7	12.7	n.d.	n.d.
0.500	+7.6	9.8	12.6	n.d.	n.d.
1.00	−0.7	3.8	9.0	120.5	94.5
10.0	+0.4	2.9	4.7	80.6	81.5
75.0	−4.6	5.0	8.5	60.6	96.4

n.d., not determined.

When the recovery (Table 1) was calculated by comparing the response of extracted spiked samples to that of direct injections of aqueous standards, it was found to be inconsistent over the evaluated concentration range. At a low concentration (1.00 ng/ml), the response of the plasma sample was 20% higher than the response of the standard; at higher concentrations (10.0 and 75.0 ng/ml), it was 20 and 40% lower, respectively. When the recovery was calculated versus blank plasma extracts that were spiked with idazoxan after the entire sample preparation, it was found to be consistent (within 15%) over the concentration range. The reason for this behaviour is not very well understood. Apparently, the introduction of endogenous plasma components into the mass spectrometer strongly influences the ionization and solvent desorption processes. However, as yet, there is no agreement on the actual phenomena taking place during the ionspray process.

The stability experiments demonstrated that idazoxan has a limited stability in plasma. When idazoxan is kept at ambient temperature in plasma, there is a marked decrease in idazoxan response at both 1.00 (−26%) and 75.0 ng/ml (−34%) after 24 h, compared to $t=0$. To illustrate this, Fig. 3A shows a regression plot of idazoxan response vs. time using a concentration of 75.0 ng/ml; it clearly indicates that plasma should be prepared as quickly as possible after the taking of a blood sample and also that sample preparation should be executed immediately after thawing of a plasma sample. The addition of 5 mg/ml of the preservative sodium fluoride did not improve this situation: after 24 h of storage at ambient temperature, the decrease at the 1.00 and 75.0 ng/ml levels was 26 and 28%, respectively.

In addition, after freezing (−20°C) and thawing of plasma samples, there was a clear trend towards deterioration of the idazoxan concentration: at 1.00 ng/ml, the response had decreased by 34% after five cycles and at 75.0 ng/ml, it had decreased by 19% (data not shown). Clearly, repeated freezing and thawing of idazoxan-containing plasma samples should be avoided as much as possible.

In the sample extract, however, idazoxan was found to be stable at 10°C for a period of at least 10 h, which is the maximum amount of time the prepared samples will be stored in the autosampler before injection into the LC system. Fig. 3B shows a regression plot of idazoxan response vs. time at a level of 1.00 ng/ml.

3.3. Application to clinical samples

To date, more than 3000 samples from clinical trials with idazoxan have been analysed using the described method. For all analytical runs, calibration curves were recorded with coefficients of correlation above 0.994. Per analytical run, quality control samples are analysed in duplicate at three levels, 1.00, 25.0 and 75.0 ng/ml. The average accuracy (expressed as the percentage bias from the nominal value) and the average precision (expressed as the percentage C.V.) found so far at these levels were +3.0 and 7.9% (1.00 ng/ml), +1.9 and 7.8% (25.0 ng/ml) and −0.2 and 5.1% (75.0 ng/ml) ($n=108$),

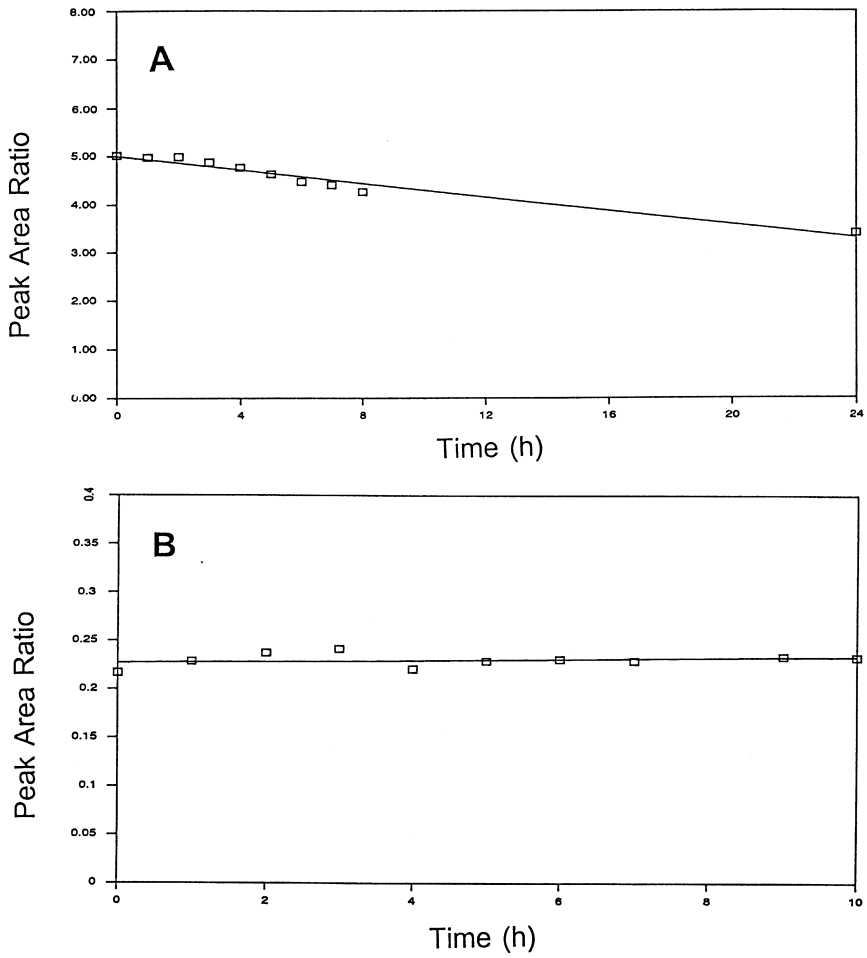

Fig. 3. Regression plots of idazoxan response vs. time for stability assessment of idazoxan; benchtop stability in plasma (ambient temperature) at 75.0 ng/ml (A) and autosampler stability in plasma extract (10°C) at 1.00 ng/ml (B).

which is consistent with the validation data presented in Table 1. Because of the short analytical run time and the rapid sample preparation procedure, a throughput of typically 150 samples per day can be achieved with this method.

Fig. 4 shows a typical plasma concentration vs. time profile for idazoxan following a relatively low single oral dose (20 mg) to a healthy volunteer. It also indicates the LLQ for the method and compares it to that of two other published methods for the determination of idazoxan, i.e. LC–UV [1] and RIA [3]. Clearly, the improved sensitivity of the LC–MS–MS method allows a more accurate evaluation of pharmacokinetic parameters after low-dose administration of idazoxan.

4. Conclusions

A straightforward and highly selective LC–MS–MS method has been developed and validated for the determination of idazoxan in human plasma. Due to the simple sample-preparation procedure, the very short analytical run time and the high sensitivity and selectivity, this method is very suitable for routine analysis in clinical trials with idazoxan. Proper care

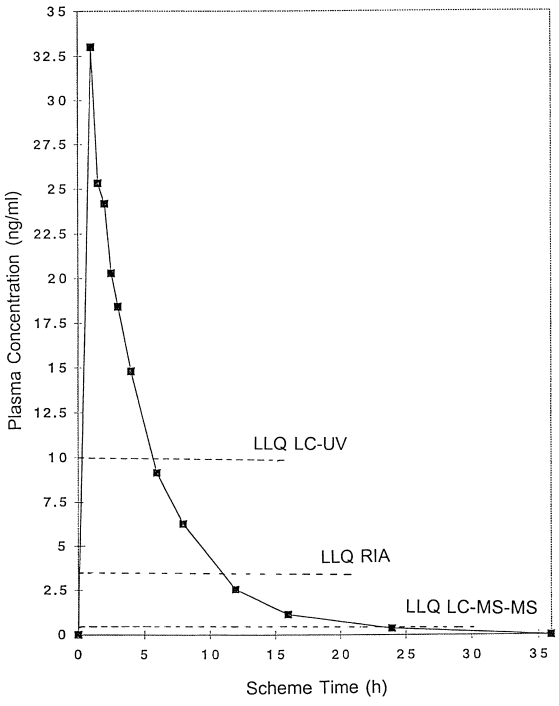

Fig. 4. Plasma concentration–time curve for idazoxan following a single oral dose of 20 mg to a healthy volunteer.

should be taken to prevent breakdown of idazoxan because of its limited stability in plasma samples.

References

[1] N.C. Muir, J.G. Lloyd-Jones, J.D. Nichols, J.M. Clifford, Eur. J. Clin. Pharmacol. 29 (1986) 743.
[2] A.C. Lane, J.D. Nichols, N.D. Steel, K. Sugden, D.S. Walter, J. Pharm. Biomed. Anal. 6 (1988) 787.
[3] A.G. Lynn, R.E. Coxon, G. Gallacher, J. Landon, J. Pharm. Biomed. Anal. 8 (1990) 685.
[4] J.D. Nichols, N.A. Hyde, K. Sugden, Analyst 114 (1989) 1215.
[5] J. Wieling, G. Hendriks, W.J. Tamminga, J. Hempenius, C.K. Mensink, B. Oosterhuis, J.H.G. Jonkman, J. Chromatogr. A 730 (1996) 381.
[6] V.P. Shah, K.K. Midha, S. Dighé, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.