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A validated high-performance liquid chromatographic assay for the simultaneous determination of denaverine and its *N*-monodemethyl metabolite in human plasma

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method for the simultaneous determination of denaverine and its *N*-monodemethyl metabolite (MD 6) in human plasma is described. The assay involves the extraction with an *n*-heptane–2-propanol mixture (9:1, v/v) followed by back extraction into 12.5% (w/w) phosphoric acid. The analytes of interest and the internal standard were separated on a Superspher RP8 column using a mobile phase of acetonitrile–0.12 *M* $NH_4H_2PO_4$–tetrahydrofuran (24:17.2:1, v/v), adjusted to pH 3 with 85% (w/w) phosphoric acid. Ultraviolet detection was used at an operational wavelength of 220 nm. The retention times of MD 6, denaverine and the internal standard were 5.1, 6.3 and 10.2 min, respectively. The assay was validated according to international requirements and was found to be specific, accurate and precise with a linear range of 2.5–150 ng/ml for denaverine and MD 6. Extraction recoveries for denaverine and MD 6 ranged from 44 to 49% and from 42 to 47%, respectively. The stability of denaverine and MD 6 in plasma was demonstrated after 24 h storage at room temperature, after three freeze–thaw cycles and after 7 months frozen storage below −20°C. The stability of processed samples in the autosampler at room temperature was confirmed after 24 h storage. The analytical method has been applied to analyses of plasma samples from a pharmacokinetic study in man.

Keywords: Denaverine; *N*-Monodemethyl denaverine

1. Introduction

Denaverine, 2-dimethylaminoethyl 2-(2-ethylbutoxy)-2,2-diphenylacetate (Fig. 1) is a neurotropic–musculotropic spasmolytic agent with additional analgesic activity [1,2]. It is used in the treatment of smooth muscle spasms of the gastroin-

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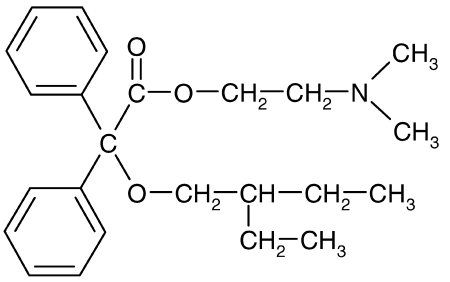
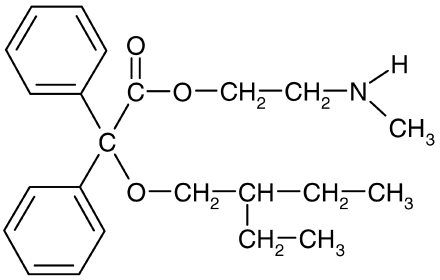
Denaverine



MD 6



Internal Standard

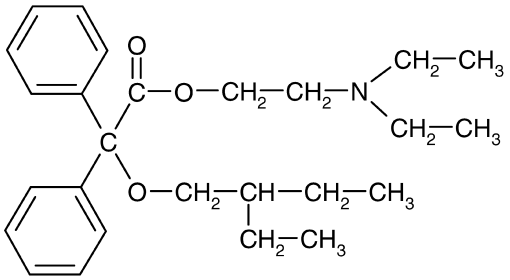


Fig. 1. Structural formulae.

testinal and urogenital tract and in the field of obstetrics [2,3]. Denaverine is available on the market as a solution for intravenous or intramuscular administration and as a suppository formulation. Although denaverine has been used successfully in therapy for more than 25 years, no information about its pharmacokinetic properties or the in vivo performance (bioavailability) of the suppository formulation has been available to date. The lack of an analytical method allowing the determination of denaverine in plasma after administration of therapeutic doses was the main reason why these areas were not covered earlier by in vivo investigations. The high-performance liquid chromatography–UV detection (HPLC–UV) method described in this paper is the first allowing the characterisation of plasma concentration vs. time curves of denaverine after administration of therapeutic doses.

Furthermore, since no information about the biotransformation of denaverine in man was available, chromatograms of plasma samples from volunteers that were dosed with denaverine were screened for possible metabolites. A metabolite of denaverine was detected and its structure was identified as the *N*-monodemethyl metabolite of denaverine [2-methylaminoethyl 2-(2-ethylbutoxy)-2,2-diphenylacetate, MD 6, Fig. 1] [4]. The activity of MD 6 is not known. However, the *N*-monodeethyl metabolite of oxybutynine, a substance that is structurally similar to denaverine, shows anticholinergic activity similar to that of the parent compound [5] suggesting that MD 6 is probably also active. Furthermore, information about the pharmacokinetics of MD 6 can help one to understand the pharmacokinetics of the parent compound. Thus, the analytical method described was validated for the simultaneous determination of denaverine and MD 6.

This paper describes the development and validation of the method according to international requirements [6,7] as well as its application to the routine determination of samples from a pharmacokinetic study.

2. Experimental

2.1. Chemicals

Denaverine HCl, MD 6 HCl and the internal standard HCl, 2-diethylaminoethyl 2-(2-ethylbutoxy)-2,2-diphenylacetate HCl (Fig. 1), were obtained from Apogepha Arzneimittel (Dresden, Germany). The purity of all three substances was ≥99%. Acetonitrile and tetrahydrofuran were of HPLC grade and were purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were also acquired from E. Merck. Blank (drug-free) human plasma was provided by the Hessen

Blood Donor Service (Frankfurt, Germany). Distilled water, prepared from demineralised water, was used throughout.

2.2. Stock solutions and standards

Stock solutions of denaverine HCl, MD 6 HCl and internal standard HCl were prepared by dissolving the accurately weighed substance in 0.1% (w/w) phosphoric acid. The working solutions were prepared by diluting the stock solutions with 0.1% (w/w) phosphoric acid to give the selected concentrations. All solutions were stored in a refrigerator between 4 and 8°C. Stock and working solutions of denaverine HCl and MD 6 HCl were shown to be stable for at least 2 years under these conditions.

2.3. Instrumentation and analytical conditions

The HPLC system consisted of a PU-980 pump, an 851-AS autosampler and a UV-975 detector (all from Jasco, Gross-Umstadt, Germany) linked to a 486DX personal computer running Nina chromatography software (Nuclear Interface, Münster, Germany).

Chromatographic separation was achieved using a Superspher RP8, 4 μm, 125 mm×4 mm column (MZ-Analysen-Technik, Mainz, Germany) with a mobile phase consisting of acetonitrile–0.12 *M* $NH_4H_2PO_4$–tetrahydrofuran (24:17.2:1, v/v), adjusted to pH 3 with 85% (w/w) phosphoric acid. Chromatography was performed at ambient temperature at a constant flow-rate of 1.5 ml/min. A total chromatography time of 12 min per sample was required. The analytes were detected by measuring their absorbance at 220 nm.

2.4. Sample preparation

After thawing at room temperature in the ultrasonic bath for 10 min, the plasma samples were shaken and centrifuged for 5 min at 2700 *g*. A 50-μl volume of internal standard solution (8.192 μg/ml) was then added to 1 ml of sample followed by 100 μl of sodium carbonate solution (1 *M*) and 2 ml of *n*-heptane–2-propanol (9:1, v/v). The samples were then shaken horizontally for 10 min (200 rpm) and centrifuged for a further 5 min (2700 *g*). After placing the samples in a freezer below −22°C until the aqueous phase was frozen (about 1 h), the organic phase was decanted into a new tube. A 100-μl volume of 12.5% (w/w) phosphoric acid was then added and samples were shaken again and centrifuged as before. The phosphoric acid extracts were then placed in conical HPLC vial inserts and an aliquot of 80 μl was injected into the HPLC system.

2.5. Quantification

Quantification was based on peak-height ratios of denaverine and MD 6 to the internal standard. Calibration curves for denaverine and MD 6 were calculated using weighted least-squares linear regression (weighting factor: $1/x^2$). All calculations were performed using Microsoft Excel 5.0 software (Microsoft Corporation, USA).

2.6. Validation

2.6.1. Specificity

The specificity of the method was verified by analysing six independent blank plasma samples (drug-free) from different volunteers. The chromatograms of these blank plasma samples were compared with chromatograms obtained after spiking the same blank plasma samples with denaverine, MD 6 and internal standard.

2.6.2. Linearity of calibration curves and lower limit of quantitation (LLQ)

To evaluate linearity, plasma calibration curves (seven concentrations, 2.5–150 ng/ml) were prepared and assayed in triplicate on 3 separate days. Graphic presentation of the calibration data together with the resulting regression line and the relative error (RE) of the interpolated concentrations (% deviation from the nominal concentrations) were used to evaluate the linearity of the calibration curves.

The LLQ of the method is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision (relative error and relative standard deviation in the ±20% range [6]). Precision and accuracy were determined as described in Section 2.6.3.

2.6.3. Accuracy and precision

Intra- and inter-day accuracy and precision were assessed by determining four plasma concentrations (two in the lower range, one in the middle range and one in the upper range of the calibration curve) sixfold on each of 3 days. The samples were prepared as a pool from a second independent weighing and stored at the same conditions as the volunteer samples (≤ -22°C). The intra- and inter-day accuracy of the method is expressed in terms of REs. The intra- and inter-day precision of the method is given by the relative standard deviations (RSDs).

2.6.4. Recovery

For the determination of the recovery of denaverine, MD 6 and internal standard, spiked plasma samples in three concentrations covering the lower, middle and upper range of the calibration curve were analysed sixfold. The peak heights of denaverine, MD 6 and the internal standard were compared to the peak heights (mean of six determinations) obtained from solutions of the analytes (see Section 2.2) injected directly onto the column.

2.6.5. Stability

The stability of denaverine and MD 6 was assessed in spiked plasma samples after storage at room temperature for 24 h, after three freeze and thaw cycles and after 7 months frozen storage below -20°C as well as in processed samples after storage in the autosampler at room temperature for 24 h. The concentrations used are detailed in Tables 2 and 3.

2.6.6. Routine analysis

The assay performance during routine analysis was monitored through the analysis of calibration samples (seven samples, 2.5–150 ng/ml) and quality control samples (duplicate samples, low, middle and upper range of calibration curves; denaverine concentrations: 4.76, 47.57 and 142.71 ng/ml; MD 6 concentrations: 4.76, 47.55 and 142.65 ng/ml) with each run. For acceptance of a run, at least four of the six quality control samples had to lie within $\pm 20\%$ of their nominal values. Two of the six QCs were allowed to be outside the $\pm 20\%$ nominal value if both were not of the same concentration [6]. Volunteer plasma samples which lay above the calibrated range were diluted with blank pool plasma to allow quantification in the validated range.

2.7. Volunteer study

After giving written informed consent, 18 young healthy Caucasian volunteers took part in a study evaluating the pharmacokinetics and bioavailability of denaverine after intravenous, oral and rectal administration of 50 mg denaverine HCl in aqueous solutions. Furthermore, the in vivo performance of the suppository formulation was characterised. The study was performed for regulatory submission and was approved by an independent ethics committee. The study was conducted in an open, randomised, changeover design with single dose administration and a wash-out phase of at least 7 days between periods. Plasma was obtained from blood samples collected prior to drug intake and up to 48 h post-dose. Plasma samples were stored frozen at less than -20°C prior to analysis.

3. Results and discussion

3.1. Method development

The assay described in this paper evolved from the evaluation and optimisation of the sample preparation, detection and chromatography of denaverine. This process began with the selection of an appropriate detection scheme. The options available for the detection of denaverine were restricted by the required LLQ of the assay (less than 5 ng/ml) and the physicochemical characteristics of the compound. Denaverine has minimal inherent fluorescence and is not electroactive at an analytically useful potential. Moreover, the compound has no functional groups available for derivatisation that could be used to enhance its detection and ester and/or ether cleavage of denaverine (producing functional groups which may be suitable for derivatisation) has been reported to be incomplete [8] and may therefore not be reproducible. Thus, no attempts were undertaken to establish a derivatisation procedure for denaverine. The UV spectrum of denaverine shows a few weakly distinct maxima between 250 and 270 nm and a significantly increased UV absorption at lower wave-

lengths. To allow the detection in the low nanogram range, the detection wavelength was set at 220 nm. The achievable LLQ of 2.5 ng/ml for both denaverine and MD 6 was fully adequate for the purposes of the pharmacokinetic study.

To establish the chromatographic separation, various analytical columns from different manufacturers filled with chemically modified silicagels (octyl, octadecyl, phenyl, nitrile materials etc.) were tested under reversed-phase conditions and were evaluated with regard to peak shape, the achievable LLQ and interferences from endogenous substances. The reversed-phase conditions applied allow the early elution of hydrophilic matrix compounds. The best results were achieved with a Superspher RP8 column in combination with a mobile phase consisting of acetonitrile–$NH_4H_2PO_4$ buffer–tetrahydrofuran adjusted to pH 3 with 85% (w/w) phosphoric acid. The use of an acidic buffer in the mobile phase allowed a reduction of the amount of organic solvent in the mobile phase whilst maintaining a runtime of 12 min.

Application of plasma precipitation procedures led to unquantifiable chromatograms, solid-phase extraction was found to be irreproducible and column switching procedures did not allow determination of the analytes in the low nanogram range. Thus, an extraction procedure consisting of two subsequent liquid–liquid extractions exploiting the pH dependent solubility of the tertiary amines denaverine and MD 6 in various solvents was developed. Two successive liquid–liquid extraction procedures were necessary, since with single liquid–liquid extraction the chromatograms showed many interfering peaks. During method development, numerous organic extraction media covering a wide range of polarity and electron donor and acceptor properties as well as different acids in different concentrations were tested for the back extraction. The mixture of *n*-heptane–2-propanol (9:1, v/v, 2 ml) in combination with phosphoric acid (12.5%) showed the best results given that a reproducible extraction rate and clean chromatograms were obtained.

3.2. Specificity

The specificity of the method was demonstrated by comparing chromatograms of plasma samples from six different volunteers – each as a blank sample and a spiked sample. No interferences with either denaverine, MD 6 or the internal standard were detected. Representative chromatograms of a blank plasma sample, a blank plasma sample spiked with denaverine, MD 6 and internal standard and a volunteer sample are shown in Fig. 2. The total run time was 12 min, the retention times of MD 6, denaverine and the internal standard were 5.1, 6.3 and 10.2 min, respectively.

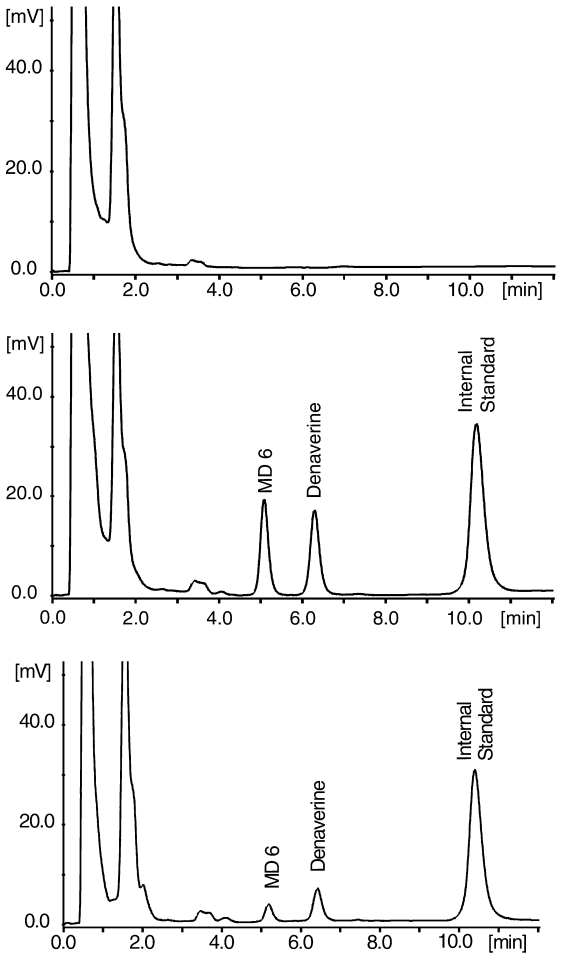


Fig. 2. Chromatograms of a blank plasma sample (top), a blank plasma sample spiked with denaverine (142.71 ng/ml), MD 6 (142.65 ng/ml), and internal standard (middle) and a volunteer plasma sample taken 1.5 h after oral administration of 50 mg denaverine HCl in an aqueous solution (bottom).

3.3. Linearity and lower limit of quantification

Visual inspection of the plotted triplicate calibration curves of denaverine and MD 6 revealed no sign of non-linearity in the concentration range 2.5–150 ng/ml. The REs of the interpolated concentrations were less than 15%. These results demonstrate the linearity of the calibration curves for denaverine and MD 6. All correlation coefficients were higher than 0.9972. The LLQ, defined as the lowest concentration of the calibration curve analysed with acceptable accuracy and precision, was 2.5 ng/ml (see Table 1).

3.4. Accuracy and precision

The overall (inter-day) accuracy of the method, expressed in terms of REs, lay between −4.8% and −3.0% for denaverine and −1.9% and 2.8% for MD 6 for the concentrations investigated (denaverine: 2.63, 5.25, 52.55 and 157.64; MD 6: 2.82, 5.63, 56.31 and 168.93 ng/ml). The overall (inter-day) precision, given by the RSDs lay between 2.8% and 5.7% for denaverine and between 3.5% and 8.1% for MD 6, respectively. Data are given in Table 1. Precision and accuracy of the method presented meet the requirements of current recommendations in bioanalytical method validation [6].

3.5. Recovery

The recoveries were between 44 (5.25 ng/ml) and 49% (157.64 ng/ml) for denaverine and between 42 (56.31 ng/ml) and 47% (5.63 ng/ml) for MD 6. The recovery of the internal standard was 44% at the concentration used in the assay (409.60 ng/ml).

Table 1
Intra- and inter-day accuracy and precision data for HPLC assay of denaverine and MD 6 in human plasma

Analyte	Nominal concentration	Intra-day (n=6)		Inter-day (n=18)	
	(ng/ml)	RE (%)	RSD (%)	RE (%)	RSD (%)
Denaverine	2.63	−3.0	5.4	−3.0	5.4
		−4.2	7.5		
		−2.3	3.4		
	5.25	−1.0	6.7	−4.8	5.7
		−6.7	3.9		
		−6.9	4.3		
	52.55	−3.8	1.2	−4.2	2.8
		−6.4	3.3		
		−2.4	1.7		
	157.64	−2.8	2.0	−3.1	3.0
		−3.8	3.9		
		−2.6	3.3		
MD 6	2.82	0.7	8.8	2.8	8.1
		2.1	9.8		
		5.3	6.2		
	5.63	−2.0	5.9	1.8	7.2
		−0.9	7.4		
		8.0	4.4		
	56.31	−6.5	2.3	−1.9	5.1
		0.8	2.8		
		0.0	5.9		
	168.93	0.1	2.6	1.1	3.5
		0.6	5.4		
		2.5	1.2		

Table 2
Stability data for denaverine (n=6 per test and concentration)

	Nominal concentration (ng/ml)	RE (%)	RSD (%)
Storage stability (≤−20°C)			
0 months	4.76	−2.3	9.5
7 months	4.76	−6.7	5.2
0 months	47.57	5.0	3.5
7 months	47.57	−1.0	2.8
0 months	142.71	8.3	2.9
7 months	142.71	0.1	3.8
Freeze–thaw stability			
0 cycles	5.25	−1.0	6.7
3 cycles	5.25	−1.9	4.3
0 cycles	157.64	−2.8	2.0
3 cycles	157.64	−3.4	2.0
Short-term stability in plasma at room temperature			
0 h	95.63	5.5	2.1
24 h	95.63	4.2	2.8
Autosampler stability			
0 h	52.55	−6.4	3.3
24 h	52.55	0.3	4.2

Table 3
Stability data for MD 6 (n=6 per test and concentration)

	Nominal concentration (ng/ml)	RE (%)	RSD (%)
Storage stability (≤−20°C)			
0 months	4.76	0.2	10.6
7 months	4.76	−4.2	4.8
0 months	47.55	2.3	5.1
7 months	47.55	−4.8	3.8
0 months	142.65	5.5	9.7
7 months	142.65	−3.2	3.5
Freeze–thaw stability			
0 cycles	5.63	−2.0	5.9
3 cycles	5.63	3.4	5.7
0 cycles	168.93	0.1	2.4
3 cycles	168.93	−3.4	4.6
Short-term stability in plasma at room temperature			
0 h	47.83	11.0	3.8
24 h	47.83	7.6	3.6
Autosampler stability			
0 h	56.31	0.8	2.8
24 h	56.31	−3.2	4.6

Table 4
Accuracy and precision of quality control samples during routine analysis

Analyte	Nominal concentration (ng/ml)	RE (%)	RSD (%)	*n*
Denaverine	4.76	−1.6	8.4	45
	47.57	−2.7	5.8	45
	142.71	−1.1	4.4	44
MD 6	4.76	−0.7	8.7	44
	47.55	−0.5	5.8	45
	142.65	−2.1	6.6	44

3.6. Stability

The stability of denaverine and MD 6 in plasma was demonstrated after 24 h storage at room temperature, after three freeze–thaw cycles and after 7 months frozen storage below −20°C. The stability of processed samples in the autosampler at room temperature was confirmed after 24 h storage. These data are summarised in Tables 2 and 3.

3.7. Routine analysis

During routine analysis, correlation coefficients for the calibration curves ($n=23$) ranged between 0.9951 and 0.9999 for denaverine and between 0.9951 and 0.9998 for MD 6. In all runs the quality control samples complied with the acceptance criteria. The summary statistics of the quality control samples shown in Table 4 confirmed the good accuracy and precision of the method.

A representative plasma concentration vs. time curve of denaverine and MD 6 obtained from a volunteer after oral administration of 50 mg denaverine HCl in an aqueous solution is shown in Fig. 3.

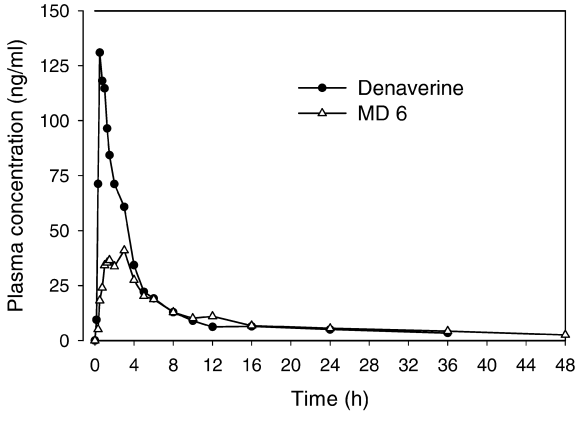


Fig. 3. Representative plasma concentration vs. time curves of denaverine and MD 6 obtained from a volunteer after oral administration of 50 mg of denaverine HCl in an aqueous solution.

4. Conclusion

The HPLC–UV method described is the first allowing the characterisation of plasma concentration vs. time curves of both denaverine and its *N*-monodemethyl metabolite in human subjects. The method fulfils all validation requirements, is simple, robust and allows a high sample throughput.

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