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Determination of tolterodine and the 5-hydroxymethyl metabolite in plasma, serum and urine using gas chromatography–mass spectrometry

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Abstract

A specific and sensitive capillary gas chromatography–mass spectrometry assay for the determination of tolterodine and the 5-hydroxymethyl metabolite (Labcode DD 01) in plasma, serum and urine is described. Extraction of the analytes was performed with liquid/liquid or solid-phase extraction prior to derivatisation with a silyl reagent. The derivatives were quantified by selected ion monitoring mass spectrometry using deuterium-labelled internal standards. A single level calibration curve was utilised for quantification of plasma, serum and urine concentrations of tolterodine and DD 01. The accuracy (inter- and intra-day) for both analytes was within 87–110% in the range 0.5 and 50 ng ml^{-1} and precision was better than 90%. Overall, this method was shown to be reliable for pharmacokinetic assays of tolterodine and the metabolite DD 01 in samples from preclinical and clinical studies. © 1997 Elsevier Science B.V.

Keywords: Tolterodine; DD 01; Gas chromatography; Mass spectrometry; Selected ion monitoring; Solid-phase extraction; Liquid/liquid extraction

1. Introduction

Tolterodine ((*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-methylphenyl)-3-phenylpropanamine) is a new muscarinic receptor antagonist intended for the treatment of urinary urge incontinence and other symptoms related to unstable bladder [1,2]. In man, tolterodine forms a pharmacologically active metabolite [3] ((*R*)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-3-phenylpropanamine; labcode DD 01), which is almost equipotent with tolterodine in vitro [4]. Thus, DD 01 has also been quantified in all clinical studies. The structures of tolterodine and DD 01 are shown in Fig. 1.

In order to evaluate the pharmacokinetic parameters of tolterodine in clinical studies a highly sensitive, selective and reliable assay was required. Moreover, a limit of quantification of 0.5 ng ml^{-1} for both tolterodine and DD 01 was necessary because of the low dosage given. The present assay describes a gas chromatographic–mass spectrometric (GC-MS) method

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and a selected ion monitoring (SIM) technique for the quantification of tolterodine and DD 01 in plasma, serum and urine. Both liquid/liquid and solid-phase extraction are described.

2. Experimental

2.1. Chemicals

Tolterodine L-tartrate (PNU-200583E; batch no. RKa 932), DD 01B (B denotes mandelate salt; PNU-200577E; batch no. RHm 922), [$^2H_5$]tolterodine L-tartrate (internal standard for tolterodine; PNU-200592E; batch no. OAR 24810) and [$^2H_5$]DD 01B (internal standard for DD 01; PNU-200593E; batch no. CA 016049) (Fig. 1) were synthesised at the Department of Medicinal Chemistry, Pharmacia & Upjohn AB, Sweden. Diethylether, methanol (HPLC grade), *n*-heptane, pyridine (AR, analytical reagent) and sodium hydroxide were obtained from Prolabo, EEC, France. Di-sodium hydrogen phosphate dihydrate, triethylammonium acetate (pH 7.0; 1 M) and phosphoric acid (85%) were supplied by Merck, Germany. Potassium hydroxide, *n*-pentane (HPLC grade), acetonitrile (Far UV) were purchased from Labscan, Ireland. *N,O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was supplied by Acros Chimica, Belgium. All aqueous solutions were prepared using Millipore water (Millipore, Canada).

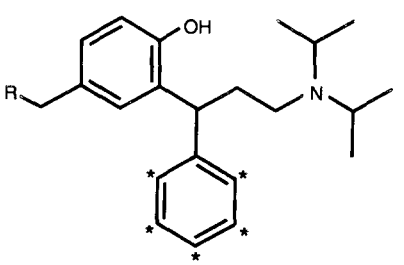
2.2. Apparatus

Quantification of the analytes was performed on a Hewlett-Packard gas chromatograph–mass spectrometer, consisting of a HP 5890 gas chromatograph equipped with an automatic injector (HP 7673A) and a mass selective detector (5970, 5971A or 5972) interfaced to the data system HP 59970C MS ChemStation (Pascal, revision 3.2) or HP-UX 59944C MS ChemStation (Unix, revision 3.2). The gas chromatographic separation was performed on a 25 m × 0.2 mm fused silica capillary column with 5% phenyl methyl silicon, 0.33 μm film thickness (HP Ultra 2, Hewlett-Packard) using helium as carrier gas. The injection port, set in splitless mode for 1 min, and the GC transfer line were operated at 250 and 280°C, respectively. The initial oven temperature was 120°C for 0.5 min and then programmed at a rate of 35°C min^{-1} to 300°C, which was maintained for 5–10 min. The mass spectrometer was operated in SIM, low resolution mode, and the electron impact ionisation was automatically set to 70 eV. The ions selected were *m/z* 382 and 387 for tolterodine and its deuterated internal standard and *m/z* 470 and 475 for DD 01 and its deuterated internal standard, respectively. Ion monitoring commenced at 6 min after injection with a duration of 3 min, and the sampling frequency (dwell time) of 30 ms for each ion was used. This enabled a total of 15–18 sampling cycles for each chromatographic peak.

Solid-phase extraction was performed with an Aspec XL sample processor (Gilson, France) using a solid-phase column of 1 ml, 100 mg C18, Isolute part No 220-0010-AA (International Sorbent Technology, UK).

2.3. Preparation of calibration standards, internal standards and quality control samples

Standards and quality control (QC) samples were prepared by spiking blank plasma, serum and urine with water solutions of different concentrations of tolterodine and DD 01. Standards



R = H Tolterodine
R = OH DD 01
* = D [$^2H_5$]tolterodine; [$^2H_5$]DD 01

Fig. 1. Chemical structures of tolterodine, DD 01 and their deuterated internal standards.

and QC samples were made from separate weighings. In order to minimise adsorption effects the final matrix solutions were prepared by adding half the volume of the matrix to the glassware before adding the analytes dissolved in water. Generally, standards and QC samples were equilibrated overnight at room temperature by mechanical agitation. After mixing and equilibration, aliquots of standards and QC samples were stored at −20°C. The calibration curve ranged from 0.5 to 50 ng ml^{-1}. Three concentrations of QC samples were used: 0.5 ng ml^{-1} (QC_{low}); 25 ng ml^{-1} (QC_{middle}); and 45 ng ml^{-1} (QC_{high}). Internal standard solution was prepared in distilled water to a concentration of 100 ng ml^{-1} with $[^2H_5]$tolterodine and $[^2H_5]$DD 01 in the same solution.

2.4. Extraction procedures

2.4.1. Liquid/liquid extraction of plasma, serum and urine samples

Aliquots (0.1–2 ml) of sample matrices were dispensed into 10 ml screw-capped glass tubes. Blank matrix was added up to approximately 1 ml to samples with a volume below 1 ml. To each tube 100 μl of the aqueous internal standard solution (equivalent to 10 ng of each of standard), 2 ml di-sodium hydrogen phosphate buffer (0.5 M; adjusted to pH 11.2 with 1 M potassium hydroxide) and 6 ml diethylether–*n*-pentane (1:3 v/v) was added. After gentle horizontal shaking for 45–60 min followed by centrifugation (1100 × *g* for 10 min at 10°C), the organic phase was transferred to another screw-capped glass tube containing 10 μl phosphate buffer (pH 11.2; 0.5 M) and subsequently evaporated to dryness under a stream of dry nitrogen at 50°C.

2.4.2. Solid-phase extraction of plasma and serum samples

Plasma and serum samples were centrifuged (1100 × *g* for 10 min) before dispensing into glass tubes to avoid clogging in the lines of the automated processor. To the dispensed samples (0.1–2 ml), 100 μl of the aqueous internal standard solution (equivalent to 10 ng of each of standard) and an aliquot of phosphate buffer (pH 7.5; 0.05 M), prepared from phosphoric acid (85%), pH-adjusted with 5 M sodium hydroxide, were added to a total volume of 4.1 ± 0.1 ml. The tubes were shaken gently for approximately 10 s and placed in the Aspec sample processor. The extraction was performed according to the following steps:

(a) column conditioning: 1 ml acetonitrile followed by 1 ml phosphate buffer (pH 7.5, 0.05 M);

(b) loading sample: 4.1 ml;

(c) washing: 1 ml distilled water followed by 1 ml acetonitrile;

(d) eluting of analytes: 1 ml methanol with 0.2% triethylamine (pH 7.0, 1 M).

The methanol phase was evaporated to complete dryness under a stream of dry nitrogen at 50°C.

2.5. Derivatisation

After the evaporation step from both procedures, 50 μl of the BSTFA reagent was added to the residue. In addition, when using solid-phase extraction, 25 μl pyridine was added. After derivatisation at 80°C for 10–20 min the excess reagent was evaporated to dryness in a stream of air or nitrogen at approximately 50°C. The residue was reconstituted with 50 μl *n*-heptane and 2 μl aliquots injected into the GC-MS system for analysis.

2.6. Quantification

Integration of the chromatographic peak and determination of the peak height was performed by Hewlett Packard data software. Three models of forming the calibration curve were used: multiple-level (unweighted and weighted; $1/x$-concentration) with 5 calibrator concentrations plus zero sample (0, 0.5, 12.5, 25, 37 and 50 ng ml^{-1}); and single-level (5 replicates of the highest calibrator concentration) drawn through the origin.

The calibration curves were calculated by means of least-square regression of the height quotients of analyte/internal standard versus the known amount of the analyte, using Hewlett Packard data software. However, this data soft-

ware could not process the weighted multiple-level calibration curve; thus, a separate laboratory data system (Nelson 2600, revision 5.2.0) had to be used for this calculation model.

All concentrations of tolterodine and DD 01 are reported as free base.

3. Results

3.1. Mass spectrometry and chromatography

The EI mass spectra of the silylated derivatives of tolterodine and DD 01 obtained at 70 eV ionisation energy are shown in Fig. 2. For SIM determination the ions m/z 382 and 387 were monitored for tolterodine and [$^2H_5$]tolterodine and m/z 470 and 475 for DD 01 and [$^2H_5$]DD 01, respectively, which all correspond to the loss of a methyl fragment from the molecular peak.

Fig. 3 shows typical chromatograms from blank and spiked human serum and urine. These mass chromatograms showed stable baselines and no impurities with the same fragments were co-eluting with the analytes.

3.2. Calibration curve parameters

Within the range 0.5 to 50 ng ml^{-1} the estimate of r^2 was better than 0.99 using unweighted or weighted multiple-level models. Unweighted multiple-level calibration resulted in a negative intercept in most cases, which led to overestimation of the QC_{low} samples. Using a single-level calibration curve drawn through the origin and with replicates ($n = 5$) of the highest calibrator, the slope was well defined and the QC_{low} value was not influenced by a variable intercept. Weighted regression analysis was also performed for comparative purposes. Table 1 shows the results of the calibration parameters, slope and intercept from five calibration curves calculated according to the three models: multiple-level regression, weighted and unweighted; and single-level regression. Fig. 4 graphically demonstrates the influence of the variable intercept on the QC_{low} results obtained from the three models of calculation.

3.3. Limit of quantification

The limit of quantification was set to 0.5 ng ml^{-1} for a 1 ml sample volume. Utilising single-level calibration the QC_{low} sample determined the limit of quantification.

3.4. Extraction recovery

Absolute recovery of tolterodine using liquid/liquid or solid-phase extraction was performed by extracting 2 ml human plasma samples spiked with 150–200 ng [^{14}C]tolterodine.

Liquid scintillation counting measurements showed that the mean recovery ($n = 5$) of [^{14}C]tolterodine in the organic phase following liquid/liquid and solid-phase extraction was 99.5% (R.S.D. 0.2%) and 86% (R.S.D. 1.1%), respectively.

3.5. Accuracy and precision

Intra- and inter-assay variations in the liquid/liquid and the solid-phase extraction procedures were determined by analysing replicates ($n = 5$) of spiked human plasma and urine samples at concentrations of approximately 0.50, 20 and 45 ng ml^{-1}. Analyses were performed on three separate days using calibration curves prepared on each day of analysis.

The intra- and inter-assay accuracy and precision obtained when using the single-level calibration model are summarised in Tables 2 and 3, respectively.

The method has been validated for both plasma and serum but the latter has been used in all clinical studies. The matrix independence of the method has been evaluated by analysing QC samples prepared in both plasma and serum versus human plasma calibration curves in the same run (Table 4).

3.6. Stability

The following stability tests were performed at concentrations of 1 and 10 ng ml^{-1}:

(a) stability in human serum at 4°C with storage time range 24–128 h and at 20°C with storage time range 12–72 h;

Table 1

Calibration curve parameters for human plasma using different regression models

Parameter	Tolterodine (run number)						DD 01 (run number)					
	1	2	3	4	5	Mean (R.S.D.%)	1	2	3	4	5	Mean (R.S.D.%)
Slope												
Multiple-level												
Unweighted[a]	0.095	0.109	0.103	0.105	0.107	0.104 (5.2)	0.103	0.119	0.119	0.128	0.121	0.118 (7.8)
Weighted[b]	0.094	0.107	0.103	0.105	0.106	0.103 (5.0)	0.101	0.117	0.117	0.124	0.122	0.116 (7.7)
Single level	0.095	0.108	0.103	0.107	0.107	0.104 (5.4)	0.101	0.120	0.120	0.124	0.123	0.118 (8.0)
Intercept												
Multiple level												
Unweighted[a]	−0.0216	−0.0489	−0.0016	−0.0152	−0.0316	−0.0238 (−75)	−0.0435	−0.0427	−0.0257	−0.0703	−0.0060	−0.0352 (−80)
Weighted[b]	−0.0057	−0.0127	−0.0094	−0.0060	−0.0044	−0.0076 (−44)	−0.0040	−0.0045	0.0053	0.0057	0.0001	0.0005 (970)

[a] Zero sample included; [b] zero sample excluded.

Table 2
Within batch (intra-assay) precision and accuracy (single-level calibration curve) of two extraction procedures for the quantification of tolterodine and DD 01 in spiked (range 0.5–50 ng) human plasma and urine samples

Sample and type of extraction	Tolterodine concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)	DD 01 concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)
	Nominal	Found (mean)			Nominal	Found (mean)		
Plasma								
Solid phase ($n = 5$)	0.51	0.52	8.5	102	0.50	0.52	2.9	104
	22.8	24.4	3.4	107	20.0	20.9	3.9	104
	45.5	47.7	4.3	105	47.5	49.3	2.0	104
Liquid/liquid ($n = 5$)	0.50	0.45	6.5	90	0.49	0.52	6.4	105
	25.0	25.1	3.1	100	24.4	23.7	2.9	97
	49.9	52.4	0.9	105	48.8	48.2	3.2	99
Urine								
Liquid/liquid ($n = 5$)	0.53	0.50	5.7	95	0.50	0.54	4.3	107
	24.2	23.7	3.8	98	24.9	26.5	4.6	107
	48.4	47.2	1.8	98	49.8	52.6	3.3	106

Table 3
Between batch (inter-assay) precision and accuracy (single-level calibration curve) of two extraction procedures for the quantification of tolterodine and DD 01 in spiked (range 0.5–50 ng) human plasma and urine samples

Sample and type of extraction	Tolterodine concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)	DD 01 concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)
	Nominal	Found (mean)			Nominal	Found (mean)		
Plasma								
Solid phase ($n = 15$)	0.51	0.51	6.9	100	0.50	0.50	8.1	100
	22.8	24.2	4.7	106	20.0	20.4	5.8	102
	45.5	49.2	5.4	108	47.5	48.9	6.3	103
Liquid/liquid ($n = 25$)	0.50	0.43	7.9	87	0.49	0.48	8.6	97
	25.0	24.1	4.3	97	24.4	23.7	6.2	97
	49.9	50.1	3.6	100	48.8	47.8	3.2	98
Urine								
Liquid/liquid ($n = 15$)	0.53	0.51	12	97	0.50	0.55	8.1	111
	24.2	23.2	5.5	96	24.9	26.0	3.9	104
	48.4	47.2	3.0	98	49.8	52.7	3.3	106

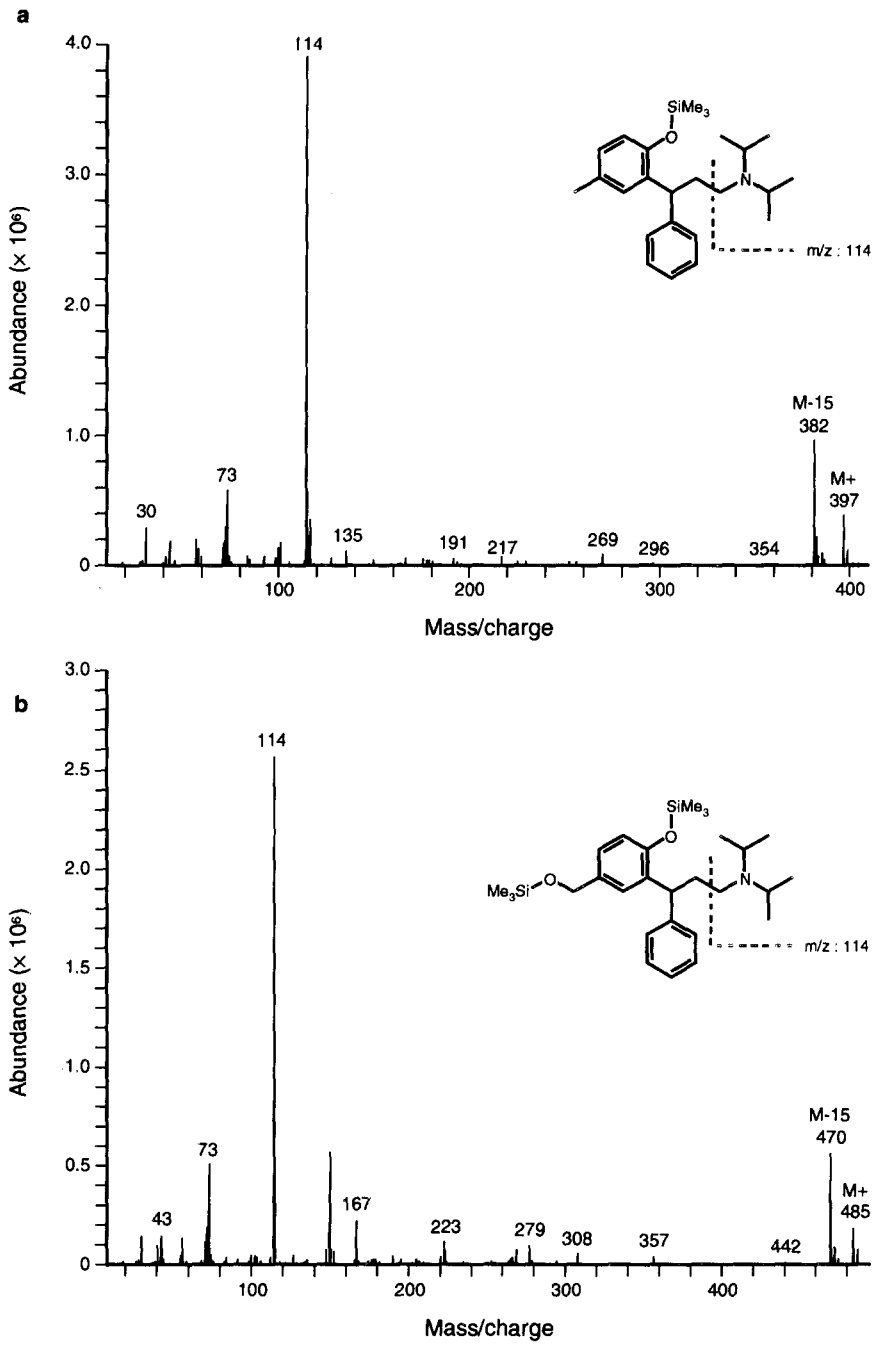


Fig. 2. Mass spectra of the tri-methylsilyl derivatives of (a) tolterodine and (b) DD 01.

(b) stability of derivatised extracted sample in heptane at 20°C with storage time range 12–72 h and at 4°C up to 14 days;

(c) stability in human serum after three freeze–thaw cycles.

The accuracy in these tests was all within the

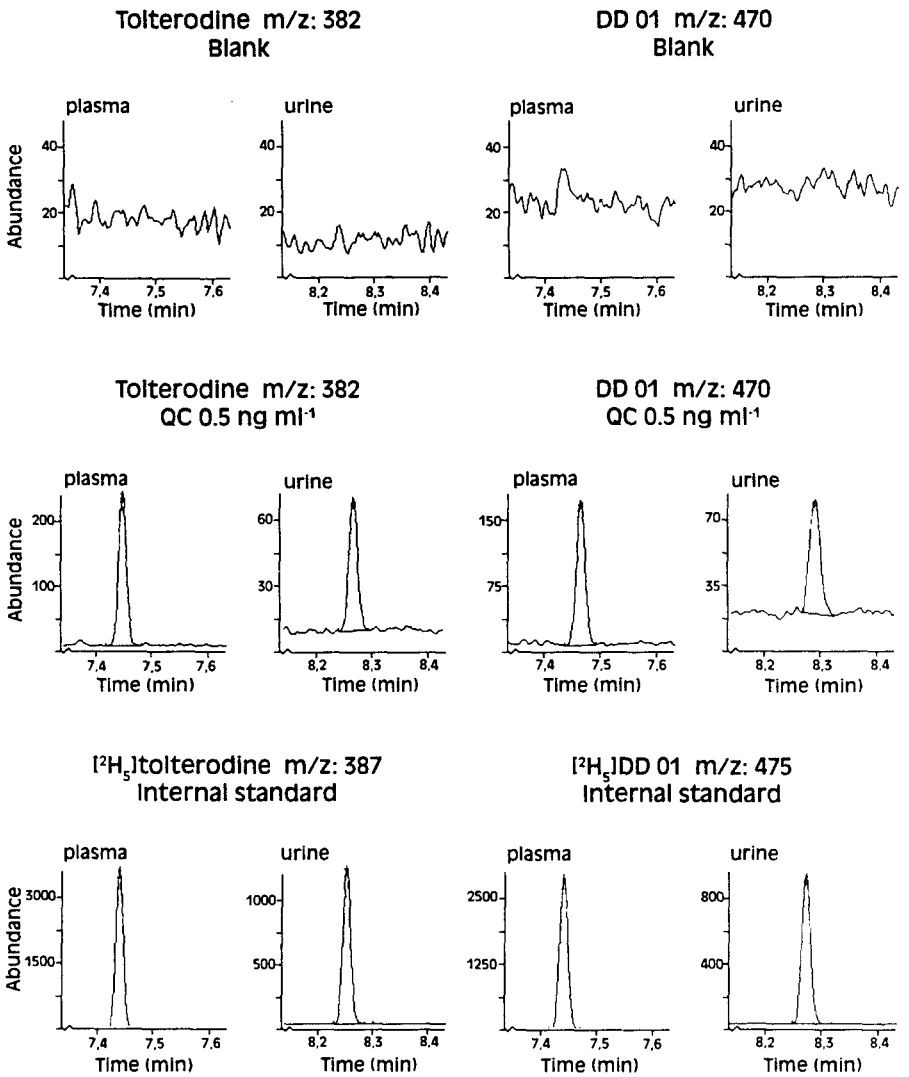


Fig. 3. Typical chromatograms from blank human plasma and urine and human plasma and urine spiked with tolterodine and DD 01 (0.5 ng ml^{-1} for both analytes) and their deuterated internal standards.

inherent variability of the method. Thus, there was no evidence of degradation of tolterodine or DD 01 under any of the test conditions.

3.7. Ruggedness

The method has been extensively used in the development programme of tolterodine and proved to be very reliable.

Measurements of human plasma QC samples prepared at the same time have been performed over a 9-month period giving 140 results of each analyte. In this series five analysts using four different mass spectrometers were involved. Fig. 5 shows a plot of all individual results of the tolterodine QC_{low} sample. Very few QC samples fell outside the acceptance criteria set for the method, $\pm 20\%$ for the QC_{low} sample. The pattern was similar for DD 01 QC_{low} samples.

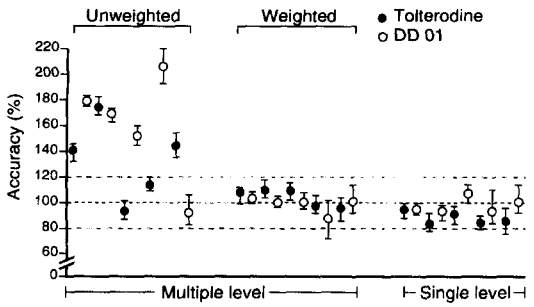


Fig. 4. Effect of the type of calibration curve and regression analysis weighting on the accuracy of the human plasma QC_{low} sample (0.5 ng ml^{-1}). Each point is the mean of 5 samples analysed during one day. Bars indicate the minimum and maximum values.

QC_{medium} and QC_{high} samples showed results well within $\pm$ 15% for both analytes. Only two analytical runs were rejected because of analyses failure of the QC samples.

4. Discussion

During the development of the liquid/liquid extraction method, a better response for DD 01 was achieved when 10 μl buffer solution was added to the empty tubes before adding the organic layer (*n*-pentane–diethylether) for evaporation. The addition of pyridine in the derivatisation step of the solid-phase extraction method slightly improved the sensitivity to DD 01 but made no difference when using the liquid/liquid extraction procedure. Possible reasons for these differences have not been further investigated.

A critical step in the assay is the derivatisation, which was shown to be dependent on the evaporation process, i.e. remaining moisture. The yield of DD 01 was more likely to be affected than that of tolterodine. This problem was more pronounced with the solid-phase extraction method, which utilised methanol as the solvent. Tolterodine was particularly susceptible to adsorption to the glassware. In order to avoid this effect the matrix was added to the glassware before the addition of aqueous solutions of the analytes and internal standards.

Unweighted regression of the multiple-level calibration curve often showed a negative intercept resulting in an overestimation of the QC_{low} sample, while the QC_{medium} and QC_{high} samples were unaffected. The variable intercept made a substantial impact on the inter-assay precision of the QC_{low} sample. In contrast, weighted regression analysis resulted in a smaller absolute value for the intercept and, accordingly, the accuracy of the QC_{low} sample approached the nominal value. The single-level mode of creating a calibration curve was shown to be a suitable alternative when a weighted regression model is not available on-line and when the unweighted multiple-level regression results in an unacceptable intercept. However, a prerequisite for the use of single-level calibration is that the zero sample must not show any de-

Table 4
Precision and accuracy for the quantification of tolterodine and DD 01 in spiked human plasma and serum samples

Sample type	Tolterodine concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)	DD 01 concentration (ng ml^{-1})		R.S.D. (%)	Accuracy (%)
	Nominal	Found (mean)			Nominal	Found (mean)		
Plasma[a] ($n = 5$)	0.75	0.74	6.1	98	0.75	0.75	3.4	100
	25.0	25.6	1.7	102	25.0	24.2	2.3	97
	45.0	46.6	1.3	103	45.0	43.3	2.0	96
Serum[a] ($n = 5$)	0.75	0.77	2.9	103	0.75	0.71	6.7	95
	25.0	25.1	2.2	100	25.0	22.9	3.0	91
	45.0	45.1	2.2	100	45.0	42.1	2.8	94

[a] Liquid/liquid extraction; weighted multiple-level calibration curve (range 0.5–50 ng ml^{-1}).

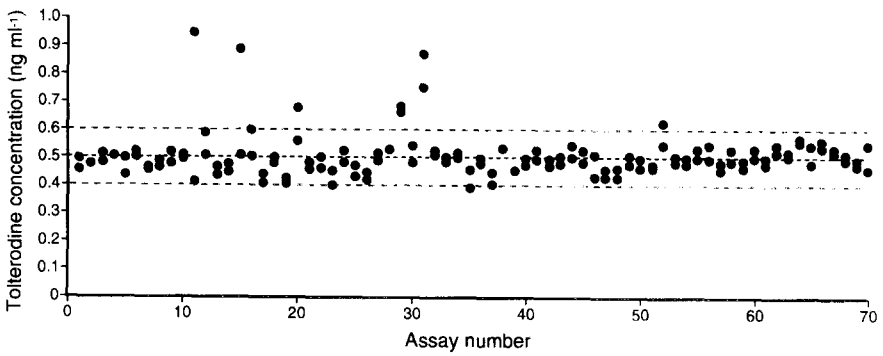


Fig. 5. Inter-assay precision and accuracy for approximately 140 QC_{low} samples of tolterodine (0.5 ng ml $^{-1}$) during a 9-month period (similar findings were reported for DD 01).

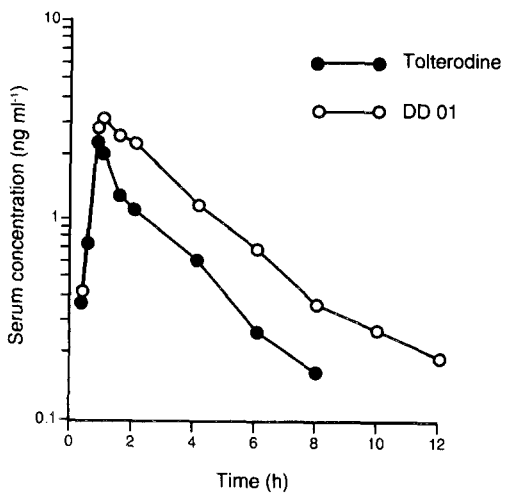


Fig. 6. Serum concentration–time curve for one volunteer receiving 2 mg tolterodine L-tartrate.

tectable levels of endogenous compounds or impurities at the retention times of the analytes or internal standards. Inherent to the use of the single-level mode is that the QC_{low} sample sets the limit of quantification.

The method described has a limit of quantification of 0.5 ng ml^{-1}. However, experiments have been made to improve the sensitivity to meet demands from clinical pharmacokinetic studies. Thus, using a single-level calibration curve and solid-phase extraction the assay has been tested down to 0.3 ng ml^{-1} for both analytes by analysing a QC_{low} sample at this concentration. The intra-($n = 5$) and inter-assay ($n = 15$) results showed an accuracy of within 97–100%, with an R.S.D. of 6–9% for both analytes. An example of a serum concentration–time curve is shown in Fig. 6.

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