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Simultaneous measurement of the major metabolites of dolasetron mesilate in human urine using solid-phase extraction and high-performance liquid chromatography

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Abstract

A method based on solid-phase extraction and high-performance liquid chromatography (HPLC) has been developed for the simultaneous quantitation of the principal active metabolites of dolasetron mesilate [i.e. MDL 74,156 (II), MDL 102,382 (III) and MDL 73,492 (IV)] in human urine. The method has been validated over the concentration range of 200–5000 pmol/ml for all three metabolites. Within-day and day-to-day coefficients of variation were less than 9 and 14%, respectively, for the three metabolites. The method allowed the simultaneous quantitation of III, IV and II and the evaluation of the urinary excretion of these metabolites in human urine following the administration of dolasetron mesilate.

1. Introduction

Dolasetron mesilate (MDL 73,147EF, 1H-indole-3-carboxylic acid, *trans*-octahydro-3-oxo-2,6-methano-2H-quinolizin-8-yl ester, methane sulphonate, I, Fig. 1) is a 5-HT_3 receptor antagonist, in development for the use in cancer patients at risk of emesis induced by cytotoxic chemo- and radiotherapy and to prevent post-operative nausea and vomiting [1,2]. It is extensively metabolized in man [3,4]. The formation of II (Fig. 1), which is the major metabolite of dolasetron in man and which is also a potent 5-HT_3 receptor antagonist and anti-emetic agent [5], is believed to result from the action of a keto-reductase [6]. After reduction of the ketone function, II is further metabolised by oxidation at the 5 and 6 positions of its indole ring to yield III and IV, respectively (Fig. 1). These metabolites also exhibited high potency and selectivity for 5-HT_3 receptors.

A previously developed method enabled the measurement of dolasetron and II in human plasma and urine [7], but not of the other more polar metabolites. Therefore, a sensitive, accurate and precise method was developed to simultaneously quantitate the urinary excretion of the three active metabolites of dolasetron mesilate in pharmacokinetic studies in man.

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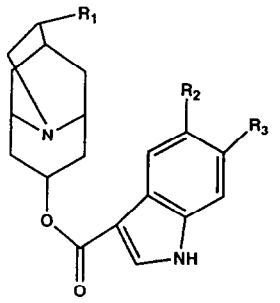

	R_1	R_2	R_3
I	=O	H	H
II	—OH	H	H
III	—OH	—OH	H
IV	—OH	H	—OH
V	—OH	CH_3	H

Fig. 1. Chemical structures of dolasetron (I), MDL 74,156 (II), MDL 102,382 (III), MDL 73,492 (IV) and the internal standard (V).

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Waters 600E multisolvent delivery system, a WISP 715 autosampler and a Waters 484 UV detector. Detection was performed at 280 nm with a range of 0.01 AUFS and a rise time of 0.3 s. The system was controlled and integration performed by a Waters Millenium chromatography manager. The column used was a 250 × 4.6 mm I.D. stainless steel pre-packed column containing 5 μm Nucleosil C_4 (Macherey-Nagel France, Strasbourg, France). A μBondapak C_{18} Guardpak pre-column (10 μm particle size, irregular, 4 mm × 6 mm I.D.) (Waters Millipore, St. Quentin Yvelines, France) protected the analytical column and was replaced after approximately 100 injections. The pre-column and the analytical column were maintained at 30°C.

Automated sample extraction was performed on a Benchmate workstation (Zymark, Paris, France) using 3 ml Sep-Pak VacCN cartridges (Waters Millipore).

2.2. Chemicals and solvents

Methanol and hydrochloric acid (37%) were of reagent grade (Merck, Darmstadt, Germany). Milli-Q water (resistance > 10 MΩ cm^{-1}) was used to prepare standard solutions. Acetonitrile was purchased from SDS (Peypin, France). Ammonium acetate, β-glucuronidase (E.C. 3.2.1.31) and 1,4-lactone saccharic acid were purchased from Sigma (St. Quentin Fallavier, France).

Eluents used in the HPLC separation were acetonitrile–0.05 *M* ammonium acetate (10:90, v/v) (solvent A), and acetonitrile–0.05 *M* ammonium acetate (60:40, v/v) (solvent B), pH in both cases adjusted to 4.7 by dropwise addition of glacial acetic acid. The eluents were filtered through a 0.2-μm Zetapore membrane (Cuno, Meriden, CT, USA) before use. The solvents were degassed by continuous helium sparging. The eluent flow-rate was 1 ml/min and a linear gradient from 100% A to 100% B over 30 min was used for separations.

The active metabolites of dolasetron, i.e. II, III and IV, were synthesized by Marion Merrell Dow Research Centre. Millimolar stock solutions were prepared with Milli-Q water for each metabolite. Working solutions were prepared with the above stock solutions to yield 100 and 10 μ*M* solutions, and stored at 4°C.

2.3. Sample hydrolysis and extraction

Three different types of samples were prepared each containing an aliquot of 100 μl of urine, 100 μl of 1 *M* sodium acetate buffer (pH 5.0) and 5000 pmol of internal standard (MDL 101,858, V, see Fig. 1). For free metabolite determination 50 μl of Milli-Q water were added; for total metabolite determination 50 μl

of β-glucuronidase also containing sulfatase activity were added and for free and sulfate conjugated metabolite determination 50 μl of β-glucuronidase and 10 μmol of 1,4-lactone saccharic acid were added.

The samples were mixed and incubated at 37°C for 90 h. This incubation time was chosen to ensure that the hydrolysis of the conjugated metabolites was complete after verification that the metabolites were stable over this period of time. The reaction was stopped by the addition of 100 μl methanol and the volume adjusted to 1 ml with Milli-Q water. The samples were centrifuged (4000 *g*, 10 min) and the supernatants were extracted on SPE cartridges. Columns were conditioned with 3 ml of methanol and washed with 2 ml of Milli-Q water. Then the supernatants were applied at the top of the solid-phase columns which were subsequently washed with 1 ml of Milli-Q water and then with 2 ml of acetonitrile. The columns were dried and compounds were eluted with 2 ml acetonitrile containing 10% of 1 *M* HCl. The samples were evaporated under a stream of nitrogen at 30°C. The residues were reconstituted in 200 μl of solvent A and 100-μl aliquots were injected onto the HPLC column.

Calibration curves were obtained by spiking blank human urine with II, III and IV at concentrations ranging from 200 to 5000 pmol/ml, the concentration of the internal standard was set at 5000 pmol/ml.

2.4. Data analysis

Concentrations of II, III and IV were determined by matching peak-height responses against a calibration curve of response ratio vs. concentration, plotted from standard sample injections. The internal standard (V) corrects for variations in the extraction efficiency and injection volume.

Peak detection, peak-height integration, peak-height ratio, calibration curve fitting (least-squares linear regression) of peak height vs. standard sample concentration were performed by the Millenium chromatography manager.

3. Results

3.1. HPLC separation

A chromatogram of a non-extracted aqueous sample is shown in Fig. 2. The retention times (capacity factors) for III, IV, II and V were 13.4 min (6.05), 13.9 min (6.32), 18.9 min (8.95) and 21.0 min (10.05), respectively. Assay selectivity was assessed by its ability to differentiate between endogenous urine peaks and the compounds of interest. Fig. 3 shows chromatograms of control urine with and without addition of III, IV, II and V in the presence of β-glucuronidase. The baseline is free of interfering peaks at the retention times of the metabolites.

3.2. Recovery

The recovery of II, III and IV was determined at two concentrations (Table 1) by extracting and injecting five replicates of the 500 pmol/ml samples and six replicates of the 2000 pmol/ml samples and comparing the peak heights with the mean peak height obtained from injections of nonextracted aqueous standard mixtures.

3.3. Linearity and limit of quantitation

The assay was calibrated over the range of 200–5000 pmol/ml. Each calibration point was prepared in duplicate. In every case a linear regression of the calibration plot resulted in a correlation coefficient better than 0.99 for each metabolite. The limit of quantitation was equal to 500 pmol/ml which is the lowest value for which accuracy and precision has been determined.

3.4. Accuracy

Accuracy was determined by calculating the difference between the mean observed value and the theoretical value as a function of the theoretical value. Relative errors (R.E.) expressed as a percentage (Tables 2 and 3) for III, IV and II were less than ± 18% at the examined concentrations.

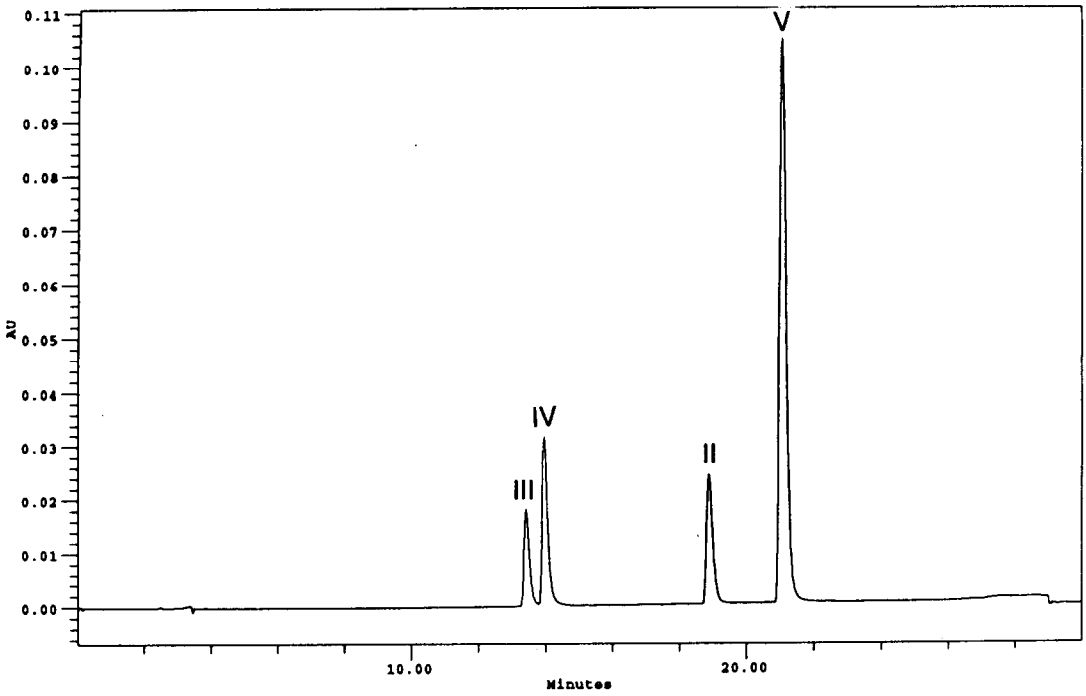

Fig. 2. Chromatographic separation of standard solutions (2000 pmol/ml) of III (13.4 min), IV (13.9 min), II (18.9 min) and V (I.S.) (21.0 min).

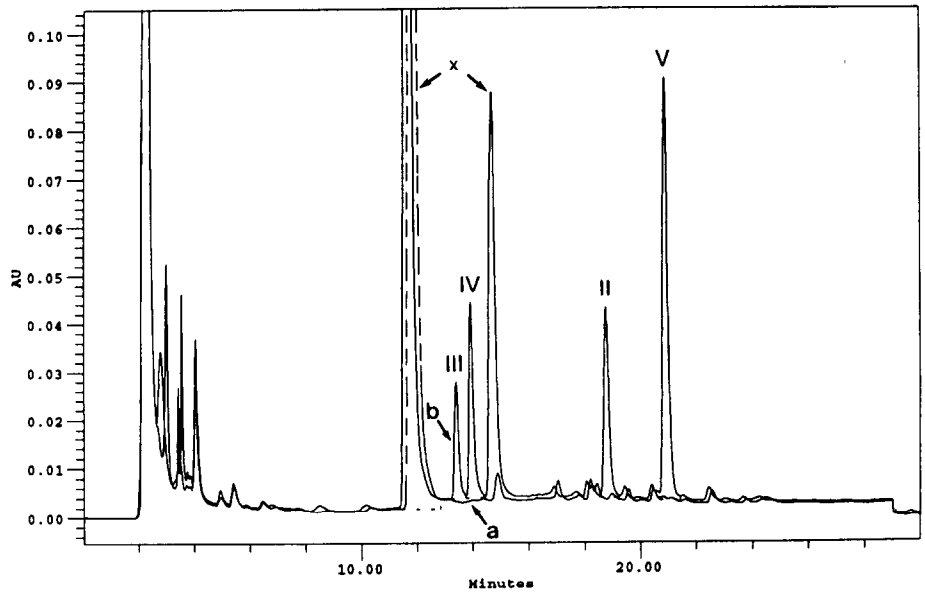

Fig. 3. Chromatographic separation of III (13.4 min), IV (13.9 min), II (18.7 min) and V (I.S.) (20.9 min) extracted from human urine. (a) Blank urine; (b) urine spiked with standards (2000 pmol/ml); x: unidentified matrix peaks.

Table 1
Recovery of the metabolites of dolasetron from urine

Compound	Recovery (mean ± S.D.) (%)	
	At 500 pmol/ml ($n = 5$)	At 2000 pmol/ml ($n = 6$)
III	66.2 ± 6.6	69.7 ± 3.7
IV	63.9 ± 7.5	66.6 ± 3.3
II	74.2 ± 5.0	72.6 ± 3.5

3.5. Precision

Precision was determined by examination of the within-day and day-to-day variations. The within-day coefficients of variation (C.V.) (Table 2), calculated by dividing the standard deviation by the mean and expressing as a percentage for III, IV and II, were less than 9% at all examined concentrations. The day-to-day coefficients of variation C.V. (Table 3) for III, IV and II were less than 14% at the examined concentrations.

3.6. Application

This method has been used to assay urine samples from a study designed to investigate the bioavailability and the effect of food on the pharmacokinetic parameters of dolasetron mesilate and its metabolites in man. Fig. 4 illustrates the urinary excretion profile of the metabolites

Table 2
Within-day accuracy and precision for the analysis of spiked urine samples

Compound	Added concentration (pmol/ml)	Urinary concentration (mean ± S.D.)($n = 5$) (pmol/ml)	C.V. (%)	R.E. (%)
III	500	470.0 ± 33.2	7.1	−6.0
	5000	5034.5 ± 176.1	3.5	0.69
IV	500	413.1 ± 35.5	8.6	−17.4
	5000	5067.2 ± 156.7	0.31	1.3
II	500	469.4 ± 18.2	3.9	−6.1
	5000	5073.4 ± 68.5	1.3	1.5

Table 3
Day-to-day accuracy and precision for the analysis of spiked urine samples

Compound	Added concentration (pmol/ml)	Urinary concentration (mean ± S.D.)($n = 11$) (pmol/ml)	C.V. (%)	R.E. (%)
III	500	466.4 ± 29.1	6.2	−6.7
	5000	4988.6 ± 124.9	2.5	−0.23
IV	500	476.6 ± 66.9	14.0	−4.7
	5000	4995.2 ± 123.6	2.5	−0.10
II	500	539.3 ± 73.0	13.5	7.9
	5000	5020.5 ± 68.8	1.4	0.41

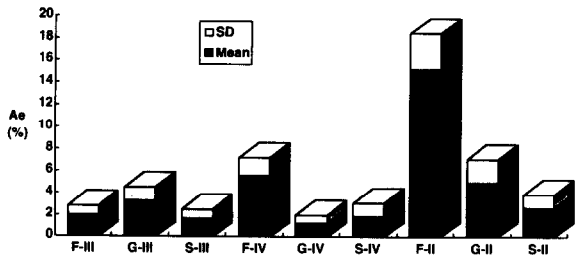

Fig. 4. Urinary excretion profile of the free (F) and glucuronide (G) and sulfate (S) conjugates of III, IV and II after oral administration of 100 mg of dolasetron mesilate to healthy male volunteers ($n = 9$).

of dolasetron mesilate given as a single oral dose of 100 mg to nine healthy male volunteers.

4. Discussion

The analytical method presented here based on solid-phase extraction and on reversed-phase HPLC was shown to be reproducible, sensitive and specific. The range evaluated for quantitating III, IV and II in human urine was from 200 to 5000 pmol/ml. The use of the automated Benchmate workstation demonstrated good reproducibility and allowed rapid sample processing. The results for urinary excretion of II in 0–24 h human urines obtained with the present method were compared to those obtained using a previously developed liquid–liquid extraction and reversed-phase HPLC method with fluorescence detection [8] (Table 4). Mean ± S.D. values were 22.6 ± 9.64% for the present method and 21.4 ± 9.50% for the method using fluorescence detection. Statistical comparison using an ANOVA test ($\alpha = 0.05$) revealed no significant differences between the two methods ($p > 0.05$).

5. Conclusion

The present HPLC method allowed the simultaneous quantitation of III, IV and II enabling the evaluation of the urinary excretion of these metabolites in human urine following the administration of dolasetron mesilate. The application of the method is illustrated by the urinary excretion profile determined after oral administration of 100 mg of dolasetron mesilate to healthy male volunteers.

Table 4
Comparison of values obtained by the present method (UV) and those obtained previously (FL) for urinary excretion of II in 0–24 h human urine expressed as the percentage of the dose administered

Subject	Intravenous		Oral fasting		Oral non-fasting	
	UV	FL	UV	FL	UV	FL
1	27.3	22.8	15.4	15.9	18.2	14.1
2	19.7	19.0	12.5	13.2	17.1	18.0
3	17.2	15.8	16.8	15.0	19.2	18.7
4	24.1	25.1	12.7	11.7	17.8	19.0
5	39.2	37.9	39.5	36.4	35.3	35.9
7	23.0	23.7	21.4	19.5	25.3	11.2
8	46.9	43.8	36.0	36.0	39.5	40.2
9	27.7	25.2	13.6	13.8	12.2	12.7
10	28.0	29.7	16.5	14.1	19.5	19.3
11	30.6	27.9	17.2	16.0	15.8	14.4
12	9.4	10.8	10.4	10.0	21.7	19.6
Mean	26.7	25.6	19.3	18.3	22.0	20.3
S.D.	10.2	9.4	9.6	9.2	8.4	9.3

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References

[1] R.C. Miller, M. Galvan, M.W. Gittos, P.L.M. van Giersbergen, P.C. Moser and J.R. Fozard, *Drug Dev. Res.*, 28 (1993) 87.
[2] M. Galvan, M.W. Gittos and M. Fatmi, *Drugs Future*, 18 (1993) 506.
[3] H. Boxenbaum, T. Gillespie, K. Heck and W. Hahne, *Biopharm. Drug Dispos.*, 13 (1992), 693.
[4] H. Boxenbaum, T. Gillespie, K. Heck and W. Hahne, *Biopharm. Drug Dispos.*, 14 (1993) 131.
[5] P.H. Boeijinga, M. Galvan, B.M. Baron, M.W. Dudley, B.W. Siegel and A.L. Slone, *Eur. J. Pharmacol.*, 219 (1992) 9.
[6] N.R. Bachur, *Science*, 193 (1976) 595.
[7] N.D. Huebert, J.J. Schwartz, C. Hinze and K.D. Haegele, *Brit. J. Clin. Pharmacol.*, 37 (1994) 120 P.
[8] N.D. Huebert, J.J. Schwartz, C. Hinze and K.D. Haegele, *Eur. J. Clin. Pharmacol.*, submitted for publication.