

## A Method to Monitor Dutasteride in Rat Plasma Using Liquid-Liquid Extraction and Multiple Reaction Monitoring: Comparisons and Validation

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**Abstract:** Three different dutasteride extraction methods were compared and a method based on liquid-liquid extraction (LLE) using methyl *tert*-butyl ether and methylene chloride was proved to be more effective than others for the extraction of dutasteride and finasteride, the internal standard (IS), from rat plasma. Additionally, a method composed of the LLE extraction, liquid chromatography, and multiple reaction monitoring (MRM) to target dutasteride and IS was validated by assessing specificity, linearity ( $r^2 = 0.9993$ , 5 - 400 ng/mL), sensitivity (the limit of detection: 4.03 ng/mL; the limit of quantitation: 12.10 ng/mL), accuracy (intra-day: 89.4 - 105.9%; inter-day: 84.9 - 100.9%), precision (intra-day: 0.8 - 6.9%; inter-day: 2.9 - 15.9%), and recovery (84.7 - 107.8%). Since the validated method was successfully applied to a pharmacokinetic study of dutasteride, it can be useful for the pharmacokinetic evaluation of newly developed dutasteride formulations.

**Keywords:** dutasteride, multiple reaction monitoring, rat plasma

### Introduction

Dutasteride, a competitive and selective inhibitor of both type I and II 5- $\alpha$ -reductases, is used for the treatment of benign prostatic hyperplasia and hair loss.<sup>1-6</sup> While its potent pharmacological activity makes it popular in clinical fields, its formulation in market is only limited to a soft gelatin capsule filled with dutasteride in oils because of its low water solubility.<sup>7</sup> Since this type of formulation generally shows issues such as limited stability, a short shelf life, and a reduced release rate of a drug, the development of various dutasteride formulations is needed.<sup>8</sup> To facilitate the development of various dutasteride formulations, it is important to have rapid and sensitive methods to analyze dutasteride as a part of their evaluation. Currently, the combination of liquid chromatography (LC) and a multiple reaction monitoring (MRM) assay (LC-MRM), a type of fast, sensitive, and specific liquid chromatography and tandem mass spectrometry (LC-MS/

MS), is mostly employed for that purpose.<sup>9-12</sup> While LC-MRM is considered as a common choice of monitoring dutasteride in a sample, there have been several options for its extraction from various plasma samples. Gomes *et al.* developed a solid phase extraction (SPE) method for its extraction from human plasma,<sup>10</sup> and Baik and Kim reported a liquid-liquid extraction (LLE) method using methyl *tert*-butyl ether and methylene chloride for the dutasteride extraction from rat plasma.<sup>11</sup> Additionally, another LLE method employing methyl *tert*-butyl ether and *n*-hexane was applied to human plasma by Contractor *et al.*<sup>12</sup> Generally, protocols for LLE are very simple, but its specificity is limited. In the case of SPE, specificity is traded off by cost as well as relative complexity in protocols.

Thus, three pre-reported methods to extract dutasteride were compared and the LLE method with methyl *tert*-butyl ether and methylene chloride was proved to be more efficient than others. Additionally, since the LC-MRM method employing the LLE method for the sample preparation had not been validated, yet, its validation was carried out. The validated method was successfully applied to a pharmacokinetic study of dutasteride and it strongly supports the possibility that the method is useful for evaluating newly developed formulations for dutasteride.

### Experimental

#### Materials and reagents

Dutasteride was obtained from MSN Laboratories Limited (India). Finasteride, an internal standard (IS) and

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HPLC grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and SPE cartridges (Strata C18-E, 50 mg, 1 mL) were purchased from Burdick & Jackson (Muskegon, MI, USA) and Phenomenex (Torrance, CA, USA), respectively. All other reagents were of analytical grade except those for HPLC.

### Conditions for liquid chromatography and tandem mass spectrometry

LC separation was performed by a LC-20 Prominence system (Shimadzu, Tokyo, Japan). The temperature of the autosampler was kept at 4°C and components in 30 µL of each sample were separated on a Hydrosphere C18 column (5 µm, 250 × 2.0 mm, YMC, Japan) at 45°C. An isocratic mobile phase condition (0.1% aqueous formic acid solution: acetonitrile, 30%: 70%, v/v) was used at a flow rate of 0.250 mL/min and the analysis time per sample was 15 minutes. The components eluted from the column were delivered into an API 2000 triple quadrupole mass spectrometer (AB/SCIEX, Foster City, CA, USA) through a Turbospray ion source (AB/SCIEX) for multiple reaction monitoring (MRM) assays of dutasteride (528.9/460.9/49, the  $m/z$  value of the precursor ion / the  $m/z$  value of the product ion / the collision energy, V) and IS (373.3/305.2/49, the  $m/z$  value of the precursor ion / the  $m/z$  value of the product ion / the collision energy, V) in positive ion mode. MS/MS spectra of dutasteride and IS are shown in Figure 1 (A) and (B), respectively. Additional mass spectrometer conditions are as follows: spray voltage at 5300 V; spray

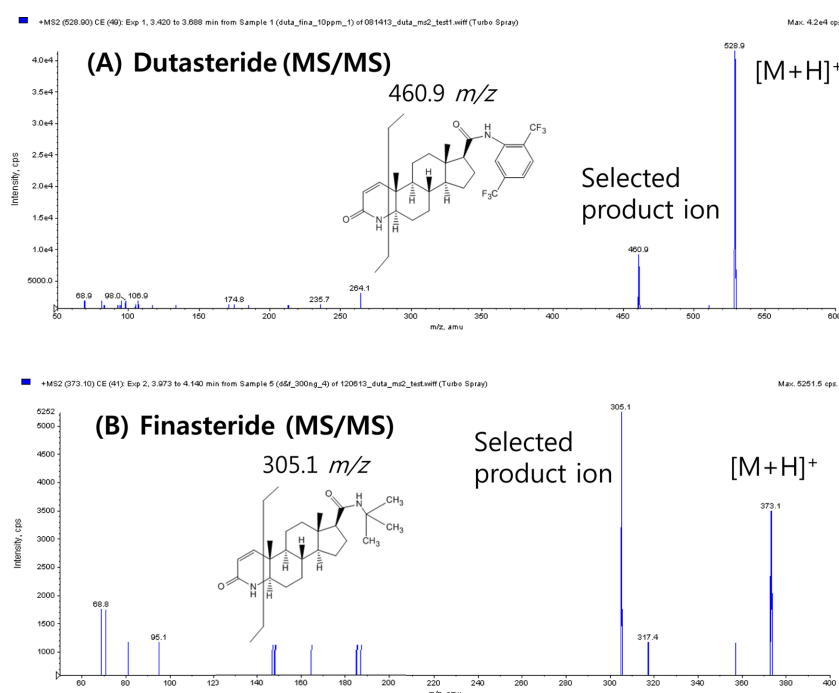
temperature at 400°C; nebulizer gas (gas 1) at 35; heater gas (gas 2) at 35; collision gas at 6; curtain gas at 18; declustering potential at 70 V; MRM transition dwell time at 100 ms; the unit Q1 resolution; the unit Q3 resolution. Analytical data were acquired by Analyst software (version 1.4.2, AB/SCIEX) and peak area ratios of dutasteride to IS were used for quantitation purpose.

### Preparation of stock solutions, standards, and quality control samples

Each stock solution (2 mg/mL) was prepared by dissolving 2 mg of dutasteride or IS in 1 mL of methanol. Working standard solutions with certain concentrations were prepared by appropriate dilution of the stock solutions. In the case of the IS working standard solution, its concentration was 200 ng/mL. Calibration standard plasma samples and quality control (QC) samples were prepared by spiking a working standard solution in blank rat plasma (final concentrations of dutasteride: 5, 10, 20, 40, 80, 160, and 400 ng/mL). All solutions were stored at -20°C until analysis.

### Extraction tests

To simplify components in calibration standard plasma samples, QC samples, and pharmacokinetic plasma samples, a certain extraction method should have been applied to a sample prior to its analysis. Thus, for the determination of the most suitable method to extract dutasteride and IS from a rat plasma sample, three different dutasteride extraction methods (SPE,<sup>10</sup> LLE1 with methyl *tert*-butyl ether and



**Figure 1.** Chemical structures and product ion spectra of dutasteride (A) and finasteride, IS (B).

methylene chloride,<sup>11</sup> LLE2 with methyl *tert*-butyl ether and *n*-hexane<sup>12</sup>) were independently applied to an extraction test plasma, a blank rat plasma spiked with dutasteride (the final concentration: 20 or 300 ng/mL) or IS (the final concentration: 20 or 300 ng/mL) as reported previously.<sup>10-12</sup> However, in the case of the SPE method, Strata C18-E cartridges instead of Strata-X DVB HL cartridges were used, because Strata-X DVB HL cartridges are not available in the market currently. Triplicate results from individual methods were compared in the aspect of recovery determined by comparing dutasteride or IS peak area of an "extracted" extraction test plasma to that of its counterpart QC working standard solution.

### Sample preparation

As a result of extraction tests, LLE1 was selected as the extraction method for calibration standard plasma samples, QC samples, and pharmacokinetic plasma samples. Briefly, 100  $\mu$ L of plasma was thawed and mixed with 20  $\mu$ L of the IS working standard solution, 100  $\mu$ L of an aqueous sodium hydroxide solution (1 mol/L), and 600  $\mu$ L of a LLE reagent (methyl *tert*-butyl ether:methylene chloride, 70:30, v/v) for 10 minutes by using a rotator. Then, the mixture was centrifuged at 12,000 rpm for 5 minutes and the resulting organic phase was dried under a gentle stream of nitrogen gas. Finally, the residue was reconstituted with 200  $\mu$ L of a 50% aqueous methanol solution for the introduction into the LC-MS/MS system.

### Method validation

The method composed of the LLE1 extraction method and the LC-MRM method were validated in terms of specificity, linearity, sensitivity, accuracy, precision, and recovery. Explanation on methods for the validation can be found in Method validation in Results and discussion.

### *In vivo* pharmacokinetic study in rats

#### *Drug administration and plasma sampling*

The comparative *in vivo* absorption studies of the drug suspension with the marketed product (Avodart<sup>®</sup>, GlaxoSmithKline) were carried out using male Sprague-Dawley rats. Marketed product and drug suspension, prepared by dispersing drug powder in a 0.2% sodium carboxymethyl cellulose solution, were administered via oral gavage at 2 mg/kg as dutasteride. Blood samples of about

0.3 mL were collected from the retro-orbital plexus at predetermined time points using heparinized tubes, and centrifuged at 12,000 rpm for 10 min. Plasma samples were then stored at -80°C until their analyses by using the LC-MRM method.

#### *Pharmacokinetic Analysis*

Collected pharmacokinetic plasma samples were treated and analyzed by using the LLE1 method and the LC-MRM method, respectively. Pharmacokinetic data analysis was performed using a BA Calc 2007 pharmacokinetic analysis computer program (Korea Food & Drug Administration, Korea). Area under the curve (AUC) was calculated using the linear trapezoidal rule by the program. Maximum plasma concentration ( $C_{max}$ ) and the time needed to reach the maximum plasma concentration ( $T_{max}$ ) were determined directly from concentration-time data.

## Results and discussion

### Comparisons of extraction methods

For the evaluation of individual dutasteride extraction method performances, the recovery of each method was determined by comparing dutasteride or IS peak area of an "extracted" extraction test plasma to that of its counterpart QC working standard solution. SPE method showed 118.4% and 120.9% recovery for IS, but much higher recovery (818.0% and 551.1%) than 100% for dutasteride (Table 1). These results are significantly different from previous ones (dutasteride recovery: 99.74 - 109.85%; IS recovery: 98.96 - 105.05%) and its reason might be related with the use of different SPE cartridges (Strata C18-E instead of Strata-X DVB HL) and the application to different species (rat instead of human).<sup>10</sup> However, since any endogenous nor exogenous interfering compound was not detected from double blank rat plasma extraction using the SPE method (data not shown), more investigation is required to find out reasons of different recovery values between the literature and the present study. In the case of LLE2 method which employs methyl *tert*-butyl ether and *n*-hexane, all recovery results showed good reproducibility (the range of coefficient of variance values: 5.2 - 14.3%), but all of them were lower than 65% (43.7 - 60.5%, Table 1). The previous report showed recovery range between 95.0% and 99.6% and the present LLE2 method application

**Table 1.** Comparisons of the extraction of dutasteride and finasteride from rat plasma using three different methods

Compound	Concentration (ng/mL)	Recovery (%), mean $\pm$ standard deviation, n = 3)		
		SPE <sup>10</sup>	LLE1 <sup>11</sup>	LLE2 <sup>12</sup>
Dutasteride	20	818.0 $\pm$ 132.4	107.8 $\pm$ 9.0	44.1 $\pm$ 6.3
	300	551.1 $\pm$ 33.1	97.6 $\pm$ 1.3	46.1 $\pm$ 2.4
Finasteride	20	118.4 $\pm$ 11.7	89.7 $\pm$ 4.3	43.7 $\pm$ 4.4
	300	120.9 $\pm$ 5.7	84.7 $\pm$ 3.8	60.5 $\pm$ 3.9

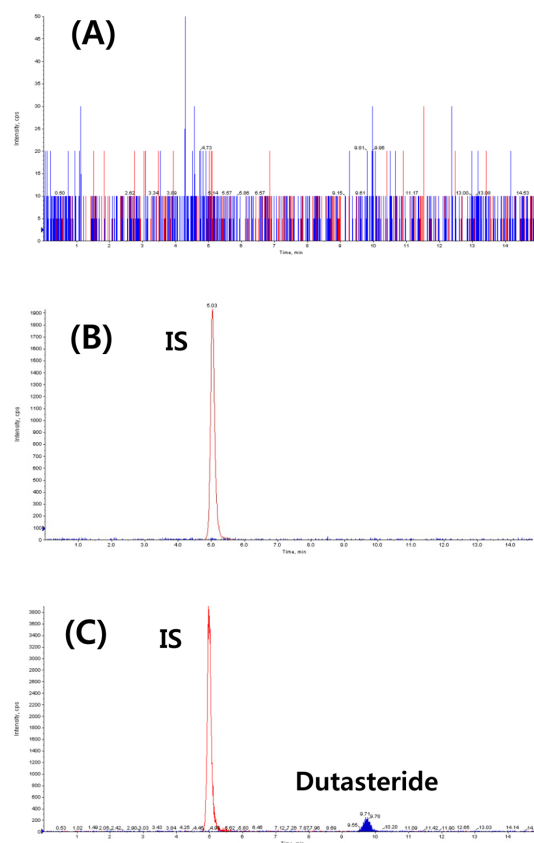
to rat plasma instead of human plasma might also be related with the difference between recovery values.<sup>12</sup> However, once again, any endogenous nor exogenous interfering compound was not detected from double blank rat plasma extraction using the LLE2 method (data not shown), and it means that more investigation is required for figuring out reasons of different recovery values between two studies. As shown in Table 1, the change of extraction solvents from methyl *tert*-butyl ether and *n*-hexane at LLE2 to methyl *tert*-butyl ether and methylene chloride at LLE1 improved recovery values (84.7 - 107.8%) and their reproducibility (1.3 - 8.3%). Thus, LLE1 method was proved to be more efficient for dutasteride and IS in rat plasma than other methods and this is the first report on the recovery information of the LLE1 method.

**Method validation**

While the LLE1 method showed good performance to extract dutasteride from rat plasma, the validation of a method to monitor dutasteride in rat plasma which includes the LLE1 extraction method has not been carried out. Thus, the method composed of the LLE1 extraction method, LC, and MRM assays for dutasteride and IS was validated by the assessment of its specificity, linearity, sensitivity, precision, accuracy, and recovery.

The specificity of the method was confirmed by the absence of peak representing dutasteride or IS from blank rat plasma samples (n = 6). While dutasteride and the IS were confirmed at 9.71 minutes and 5.04 minutes, respectively, from the positive control analysis, any endogenous nor exogenous interfering compound peak couldn't be observed from blank plasma analyses (Figure 2). The good linearity ( $r^2 = 0.9993$ ) of the method was also observed at a dutasteride concentration range between 5 - 400 ng/mL, and the limit of detection (LOD, S/N ≥ 3) and the limit of quantitation (LOQ, S/N ≥ 10) were calculated as 4.03 ng/mL and 12.10 ng/mL, respectively. Its precision and accuracy were studied by using QC samples (n = 5) at 5 different concentrations (5, 10, 40, 160, and 400 ng/mL). Accuracy and precision were expressed as the percentage of a calculated concentration based on a calibration equation to its theoretical concentration and the coefficient

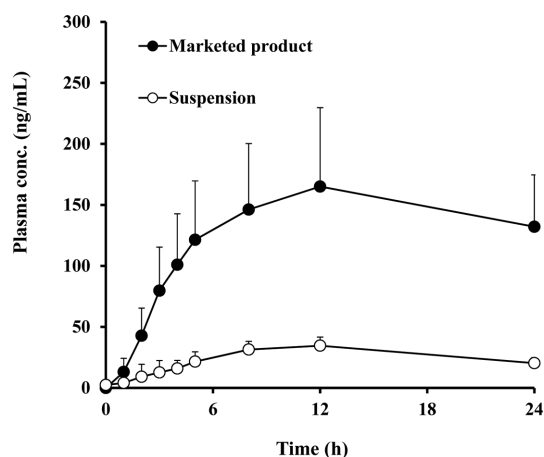
of variance value among replicate results, respectively. Intra-day comparisons were done within a day and inter-day comparisons were carried out over five consecutive days. As shown in Table 2, the intra-day accuracy, the inter-day accuracy, the intra-day precision, and the inter-day precision were determined as 89.4 - 105.9%, 84.9 - 100.9%, 0.8 - 6.9%, and 2.9 - 15.9%, respectively. As mentioned above (Comparisons of extraction methods in Results and discussion), the recovery range of the method was determined as 84.7 - 107.8% (Table 1).



**Figure 2.** Multiple reaction monitoring chromatograms of double blank rat plasma (A), blank rat plasma (B), dutasteride (10 ng/mL) plasma standard with IS (finasteride, 20 ng/mL) (C)

**Table 2.** Intra-day and inter-day comparisons of results from LC-MRM analyses of dutasteride in rat plasma

Theoretical concentration (ng/mL)	Intra-day comparisons			Inter-day comparisons		
	Calculated concentration (ng/mL)	Accuracy (%)	Precision (coefficient of variance, %)	Calculated concentration (ng/mL)	Accuracy (%)	Precision (coefficient of variance, %)
5	5.29	105.9	6.9	4.24	84.9	15.9
10	9.40	94.0	0.8	9.05	90.5	9.9
40	36.74	91.8	3.4	40.34	100.9	2.9
160	142.97	89.4	2.0	158.26	98.9	4.3
400	360.54	90.1	2.2	388.01	97.0	4.7



**Figure 3.** The blood concentration of dutasteride from drug suspension (○) and marketed product (Avodart<sup>®</sup>, ●) in Sprague-Dawley rats. Data represent mean ± standard deviation (n = 10).

**Table 3.** The pharmacokinetic parameters of dutasteride from the drug suspension and marketed product (Avodart<sup>®</sup>) after a single oral administration in rats at a dose of 2 mg/kg

Pharmacokinetic Parameters	Drug suspension	Marketed product
AUC <sub>(0-24h)</sub> (ng·h/mL) <sup>1)</sup>	581.0 ± 108.0	3136.7 ± 1065.2
C <sub>max</sub> (ng/mL) <sup>1)</sup>	36.0 ± 8.0	173.9 ± 57.5
T <sub>max</sub> (h) <sup>1)</sup>	9.6 ± 2.2	14.0 ± 5.42
Relative bioavailability <sup>2)</sup>	–	5.4

<sup>1)</sup>Data are expressed as the mean ± standard deviation (n=10).

<sup>2)</sup>Calculated as percentage of the mean AUC<sub>(0-24h)</sub> of Avodart<sup>®</sup> to drug suspension.

#### **In vivo pharmacokinetic study**

The validated method was applied to pharmacokinetic studies in rats to determine the drug concentration in rats after oral administration. Figure 3 presents the plasma concentration-time profile of the 5- $\alpha$ -reductase inhibitor in rats after oral administration of drug suspension and marketed product at a dose equivalent to 2 mg/kg. And the pharmacokinetic parameters, including T<sub>max</sub>, C<sub>max</sub> and AUC<sub>(0-24 h)</sub>, are listed in Table 3. In both formulations, the plasma concentration of dutasteride was gradually increased and peaked 12 h after *per oral* administration. The intestinal drug absorption of Avodart<sup>®</sup> was significantly higher than drug suspension, providing 5.4-fold higher AUC<sub>(0-24h)</sub> value compared to drug suspension, used as a reference. The C<sub>max</sub> value of Avodart<sup>®</sup> was also higher compared to drug suspension, revealing about 4.8-fold higher value. It suggests that the oily marketed product spontaneously formed fine oil droplets after digestion by lipase in the gastrointestinal track and present the drug in a

dissolved form, avoiding the dissolution step. These pharmacokinetic data analyzed by the validated method are expected to be useful for next formulation study for developing novel oral dosage form of dutasteride.

#### **Conclusions**

Three pre-reported methods for dutasteride extraction were compared and the LLE method using methyl *tert*-butyl ether and methylene chloride was proved to be more efficient than others for its extraction from rat plasma. Since the LC-MRM method applied to samples prepared with the LLE method had never been validated, yet, its validation was also conducted. A basic pharmacokinetic study on dutasteride was successfully carried out using the validated method, and it strongly supports that the method can be useful for evaluating new formulations for dutasteride.

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#### **References**

- Gisleskog, P. O.; Hermann, D.; Hammarlund-Udenaes, M.; Karlsson, M. O. *Br. J. Clin. Pharmacol.* **1999**, 47, 53.
- Evans, H. C.; Goa, K. L. *Drugs Aging* **2003**, 20, 905.
- Gaines, K. K. *Urol. Nurs.* **2003**, 23, 218.
- Brown, C. T.; Nuttall, M. C. *Int. J. Clin. Pract.* **2003**, 57, 705.
- Olsson, G. P.; Hermann, D.; Hammarlund-Udenaes, M.; Karlsson, M. O. *Eur. J. Pharm. Sci.* **1999**, 8, 291.
- Olsen, E. A.; Hordinsky, M.; Whiting, D.; Stough, D.; Hobbs, S.; Ellis, M. L.; Wilson, T.; Rittmaster, R. S.; Dutasteride Alopecia Research Team. *J. Am. Acad. Dermatol.* **2006**, 55, 1014.
- U. S. Food Drug Administration, "Application Number: 21-319." <[http://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2001/21319\\_Duagen\\_biopharmr\\_P1.pdf](http://www.accessdata.fda.gov/drugsatfda_docs/nda/2001/21319_Duagen_biopharmr_P1.pdf)>, cited 27 Jun, 2012.
- Ofner, C. M. 3rd.; Zhang, Y. E.; Jobeck, V. C.; Bowman, B. J. *J. Pharm. Sci.* **2001**, 90, 79.
- Ramakrishna, N. V. S.; Vishwottam, K. N.; Puran, S.; Koteswara, M.; Manoj, S.; Santosh, M. *J. Chromatogr. B* **2004**, 809, 117.
- Gomes, N. A.; Pudage, A.; Joshi, S. S.; Vaidya, V. V.; Parekh, S. A.; Tamhankar, A. V. *Chromatographia* **2009**, 69, 9.
- Baek, I. -H.; Kim, M. -S. *Chem. Pharm. Bull.* **2012**, 60, 1468.
- Contractor, P.; Kurani, H.; Guttikar, S.; Shrivastav, P. S. *Biomed. Chromatogr.* **2013**, 27, 1168.