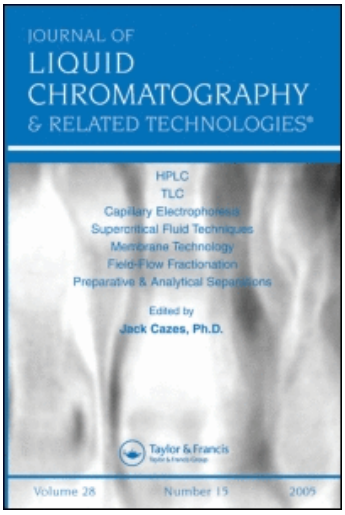

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Analytical HPLC Method Validation of Amiloride and Its Pharmacokinetic Study in Humans

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Abstract: This study was aimed to validate a reliable analytical method for the pharmacokinetic study of amiloride in human plasma by a high performance liquid chromatography (HPLC) system with UV detection. Triamterene was used as an internal standard. After extraction with ethylacetate, the supernatant was evaporated. Then, the residue was reconstituted and an aliquot was injected onto the HPLC system. Separation was performed on a Capcell Pak C_{18} UG120 column (4.6 mm × 150 mm, 5 μm particles) with a mobile phase of 12% acetonitrile containing 0.4% glacial acetic acid and UV detection at a wavelength of 360 nm. The intra- and inter-day precision expressed as the relative standard deviation was less than 15%. The flow rate of mobile phase was 1 mL/min and the retention time of amiloride and internal standard, triamterene, was found to be 3.06 and 8.80 min, respectively. The lower limit of quantification (LOQ) was 0.2 ng/mL of amiloride using 1 mL of plasma. The calibration curve was linear in the concentration range of 0.2–50 ng/mL ($r^2 = 1.0000$). The mean accuracy was 85.1–101.7%. The coefficient of variation (precision) in the intra- and inter-day validation was 2.1–12.5 and 2.4–13.7%, respectively. The pharmacokinetics of amiloride was evaluated after a single oral administration of 10 mg to healthy volunteers. The AUC_{0-72hr}, C_{max}, T_{max}, and $T_{1/2}$ were 267.7 ± 60.0 ng · hr/mL, 22.9 ± 5.4 ng/mL, 3.0 ± 0.8 hr, and 13.6 ± 2.4 hr, respectively. These results

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demonstrated that this method was highly feasible and reproducible for pharmacokinetic studies of amiloride in eight volunteers after oral administration (10 mg as amiloride HCl).

Keywords: Amiloride, HPLC, Human plasma, UV detection

INTRODUCTION

Amiloride (N-amidino-3,5-diamino-6-chlropyrazine-2-carboxamide) (Figure 1a) is a K^+-sparing diuretic by competitively inhibiting the epithelial Na^+ channel of collecting duct principal cells. It is used to restore normal serum K^+ levels in patients who develop hypokalemia, and in patients who would be exposed to a particular risk if hypokalemia were to develop.[1]

Studies on the pharmacokinetics of amiloride are rather poorly documented, and the paucity of data on disposition of this drug is mainly due to the lack of sensitive and specific analytical methods for its measurement in biological fluids. Several studies have reported that peak plasma levels occur 4 hr after oral administration, absorption ranges from 90% to 95% of the dose and half-life ranges from 10–14 hr.[2–5] Since amiloride exhibits an extremely low therapeutic range (0.5–25 ng/mL), only a few sufficiently specific and sensitive HPLC methods for pharmacokinetic studies have been described.[6–11]

The pharmacokinetics and bioavailability of amiloride in plasma and/or urine have been investigated by determining its levels by

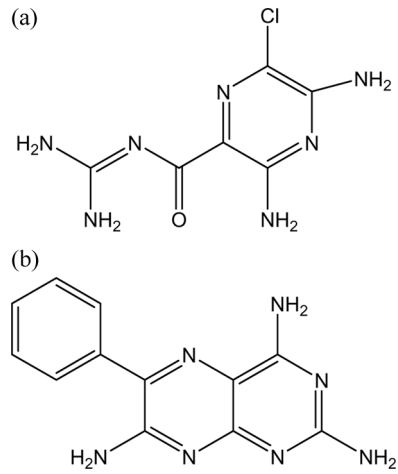

Figure 1. Chemical structures of (a) amiloride and (b) triamterene.

fluorometric,[12] radioactive,[13] densitofluorimetric,[14] or spectrometric[15] assays. High performance liquid chromatographic (HPLC) methods for determining amiloride in plasma and/or urine have also been reported.[6,16–19] These methods are selective and sensitive, but they require time consuming extractions or mobile phase being saturated with stationary phase. More simplified HPLC techniques employing solid phase extraction and precolumn enrichment procedures have been published.[7,9] Recently, more sensitive LC/MS/MS method were published.[20] However, as compared with the HPLC-UV method commonly used, this method is more sophisticated and much more expensive.

Thus, the present study reports a sensitive, rapid, and simplified reversed phase HPLC method for determining amiloride in human plasma with improving the limit of quantification (LOQ) to 0.2 ng/mL. This method has been validated, and was used to determine amiloride in the plasma of eight volunteers after oral administration of amiloride, providing data on the pharmacokinetics and bioavailability of the drug.

EXPERIMENTAL

Material

Amiloride hydrochloride and triamterene were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were analytical grade and used without further purification.

Preparation of Standards and Quality Control Samples

Stock solutions of amiloride (0.5 mg/mL) were made by dissolving in distilled water and diluted with 45% methanol solution (0.1 M $HClO_4$: methanol = 55 : 45, (V/V)) to concentration of 2, 5, 10, 50, 100, 200, and 500 ng/mL. Standard solutions of amiloride in blank human plasma were prepared by spiking the appropriate volume of various diluted stock solutions giving trial concentrations of 0.2, 0.5, 1, 5, 10, 20, and 50 ng/mL. Triamterene, as an internal standard (IS) was dissolved in methanol to make a stock solution (50 μg/mL). The working IS solution (500 ng/mL) was prepared by diluting the stock solution with 45% methanol solution (0.1 M $HClO_4$: methanol = 55 : 45, (V/V)). The chemical structure of triamterene is shown in Figure 1(b). The Quality control (QC) samples, at a concentration 0.5 ng/mL (low) and 20 ng/mL

(high) were made by diluting the working solution with blank human plasma.

Preparation of Samples

The samples were stored in the freezer at −70°C and allowed to thaw at room temperature before processing. Each 1 mL of plasma, 50 μL of internal standard (500 ng/mL of sodium triamterene), and 0.5 mL of 5 M NaOH were added to a glass tube. After brief vortex mixing for 10 sec, 6 mL of ethylacetate was added and the mixture was vortex mixed for 1 min. After centrifugation at 2,500 rpm for 10 min, the organic layer was transferred to a new glass tube and evaporated to dry under a gentle stream of nitrogen at 60°C. The residue was reconstituted with 400 μL of mixture of 0.1 M $HClO_4$ and methanol (55:45, v/v) and a 100 μL aliquot was injected into the HPLC system.

Apparatus and Chromatographic Conditions

The determination of amiloride was carried out using an HPLC system consisting of a Waters 515 HPLC Pump, Waters 717 Plus Autosampler, equipped with Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA), and Autochro Win-Chromatography Data System (Young Lin Instrument Co., Ltd., Anyang, Korea). The separation was performed on a Capcell Pak C_{18} UG120 column (4.6 mm × 150 mm I.D., 5 μm, Shiseido, Tokyo, Japan). The column temperature was 30°C. Mobile phase was a 12% solution of acetonitrile containing 0.4% glacial acetic acid, with pH adjusted to 4.5 with triethylamine, and the signals were monitored with UV detection at a wavelength of 360 nm. The flow rate was 1.0 mL/min.

Validation of the Method

Evaluation of the reversed-phase HPLC method was based on proportionality (linearity assay), precision, and accuracy.

Specificity

Drug free blank human plasma was tested for interference using the proposed HPLC method, and the result was compared with those from human plasma spiked with amiloride and internal standard.

Linearity

The calibration curve consisted of the seven concentrations; 0.2, 0.5, 1, 5, 10, 20, and 50 ng/mL of amiloride. The calibration curves were obtained by linear regression; the ratio of amiloride peak area to internal standard peak area was plotted vs. amiloride concentration in ng/mL.

Precision and Accuracy

The intra- and inter-day precision (coefficients of variation, CV%) and inter-day accuracy (bias%) of the assay procedure were determined by the analysis of five samples at each concentration in the same day and one sample at each concentration in 5 different days, respectively.

Sensitivity

The limit of quantification (LOQ) was defined as the lowest concentration at which the precision expressed by CV% was lower than 20%, the accuracy expressed as bias% was within 80–120% and ratio of signal to noise was better than 5.

Recovery

The recovery was calculated by comparing the peak areas of the samples extracted with those obtained from a mobile phase with the same concentration.

Stability

Freeze and thaw stability

After subjecting to three freeze (stored at −20°C for 24 hr) and thaw cycles, these plasma samples were analyzed by HPLC.

Short term stability

After QC samples were exposed to room temperature for 4 hr, these samples were analyzed by HPLC.

Long term stability

After QC samples were stored in the deep freezer at −70°C for 4 weeks, these samples were analyzed by HPLC.

Standard solution stability

After stock solutions of amiloride (0.5 mg/mL) and internal standard (50 μg/mL) were left at room temperature for 6 hr, these solutions were analyzed by HPLC.

Processed sample stability

After QC samples were left in the autosampler at ambient temperature (ca. 20°C) for 5 hr, these samples were analyzed by HPLC.

Preparation of Biological Samples

The validated method was applied to evaluate the bioavailability of amiloride. According to medical history, physical examination, and standard laboratory test results (blood cell count, biochemical profile, and urinalysis), eight (8 male) healthy volunteers were selected for this study. The demographic data of these volunteers were; mean age 23.8 years, mean height 173.5 cm and mean weight 68.9 kg.

After an overnight fast, a predosing plasma sample was collected. Each volunteer was then orally administered two capsules (5 mg as amiloride hydrochloride), namely Amilo® tablet 5 mg (Kuhnil Pharm. Co. Ltd., Seoul, Korea) with 240 mL of water. The volunteers continued to fast for 4 hr, after which a standard lunch was served. Plasma samples were collected before administration and at designated time intervals, i.e. 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 48, and 72 hr post dosing. The plasma collected was stored at −70°C until assayed for the amiloride content. The pharmacokinetic parameters were calculated by bioavailability analytical program, BA Calc 2002.[21]

RESULTS AND DISCUSSION

High-Performance Liquid Chromatography

Selectivity

Drug free human plasma was screened and no endogenous interference was observed at the retention time of amiloride and internal standard. The Capcell Pak UG C_{18} column has been used as an analytical column since it has 80 Å pore size, which limits the access of large molecules such as proteins, retain drug molecules longer, and improved sensitivity.[22]

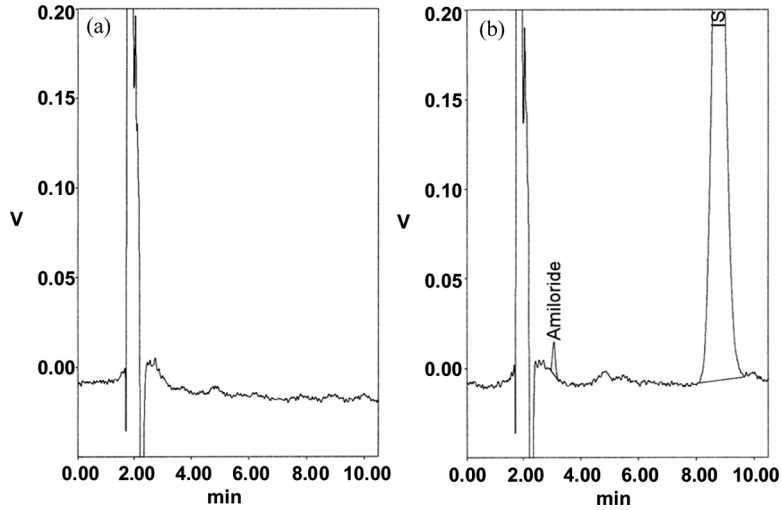

Figure 2. Representative chromatograms of (a) blank plasma and (b) blank plasma spiked with amiloride (0.5 ng/mL) and triamterene as an internal standard (IS, 500 ng/mL).

Representative chromatograms of an extracted blank human plasma sample for blank (Figure 2a) and one containing 0.5 ng/mL of amiloride and 500 ng/mL of internal standard (Figure 2b) were illustrated.

Linearity

The calibration curves were linear in the studied range. The mean equation of the calibration curve consisting of seven points was $y = 0.0191x + 0.0007$ with correlation coefficient of $r^2 = 1.0000$, where y represents the peak area ratio of amiloride to internal standard and x represents the amiloride concentration in ng/mL.

Precision and Accuracy

The intra- and inter-day precision and accuracy results are shown in Table 1. The values obtained were lower than the limits required for biological samples, ±20% for the precision and inaccuracy of the lower limit of quantification (0.2 ng/mL) and ±15% for the both of the other concentrations. The mean accuracy was 85.1–101.7%. The precision in the intra- and inter-day validation was 2.1–12.5, and 2.4–13.7%, respectively.

Table 1. Reproducibility of amiloride determination in human plasma ($n = 5$)

Amiloride concentration (ng/mL)	Precision (CV%)		Accuracy
	Intra-day	Inter-day	
0.2 (LOQ)	8.2	13.7	85.1
0.5	7.2	9.5	93.0
1	12.5	7.9	98.7
5	5.3	2.5	96.3
10	2.1	3.2	100.8
20	2.4	2.5	101.7
50	2.2	2.4	99.8

Sensitivity

The LOQ of amiloride was 0.2 ng/mL. As compared with a previous report,[18] this method was sufficiently sensitive, with a quantification limit lower than the minimum concentration recommended for plasma samples obtained after the administration of 5 mg amiloride. Moreover, the LOQ of amiloride was comparable with a published method using more expensive and sophisticated LC/MS/MS (0.1 ng/mL).[20]

Recovery

The extraction recoveries of amiloride at 0.5 ng/mL and 20 ng/mL were $63.6 \pm 9.1\%$ and $65.4 \pm 0.1\%$, respectively. A recovery of $90.7 \pm 1.8\%$ was obtained for the IS.

Stability

The results are shown in Table 2. Freezing and thawing did not reveal any detrimental effects on the absolute concentrations of analyte spiked to human plasma. After completion of three freezing and thawing cycles, the measured concentrations of amiloride still ranged between 100.2 and 101.6%. Short term and long term stability was ranged from 99.4 to 102.3% and 99.6 to 100.2%, respectively. In addition, any obvious trends of concentration changes over time were not observed in standard solution stability (99.9% for 0.5 mg/mL amiloride and 99.7% for 50 μg/mL internal standard) and processed sample stability. Thus, this assay method for the determination of amiloride in human plasma has a sufficient stability in human plasma for the bioavailability assessment of amiloride.

Table 2. Stability of amiloride determination in human plasma ($n = 3$)

Amiloride concentration (ng/mL)	Freeze-thaw stability (%)	Short term stability (%)	Long term stability (%)	Processed sample stability (%)
0.5	101.6	99.4	99.6	107.9
20	100.2	102.3	100.2	100.4

Application to the Bioavailability of Amiloride

Representative chromatograms of an extracted predose plasma sample containing 500 ng/mL of internal standard (Figure 3a) and plasma sample collected at 24 hr after oral administration of 10 mg to human subjects (Figure 3b) were illustrated. The mean plasma amiloride concentration time profile after a single dose of two Amilo® tablet is shown in Figure 4. The pharmacokinetic parameters of amiloride was $267.7 \pm 60.0\,\text{ng} \cdot \text{hr/mL}$ of $AUC_{0\text{–}72hr}$, $22.9 \pm 5.4\,\text{ng/mL}$ of C_{max}, $3.0 \pm 0.8\,\text{hr}$ of T_{max}, and $13.6 \pm 2.4\,\text{hr}$ of $T_{1/2}$, which were very similar to the previous reports.[23–27] Moreover, as compared with previously reported liquid chromatographic determination of amiloride, this method improved the lower limit of quantification and presented the pharmacokinetic data of 10 mg of amiloride hydrochloride.

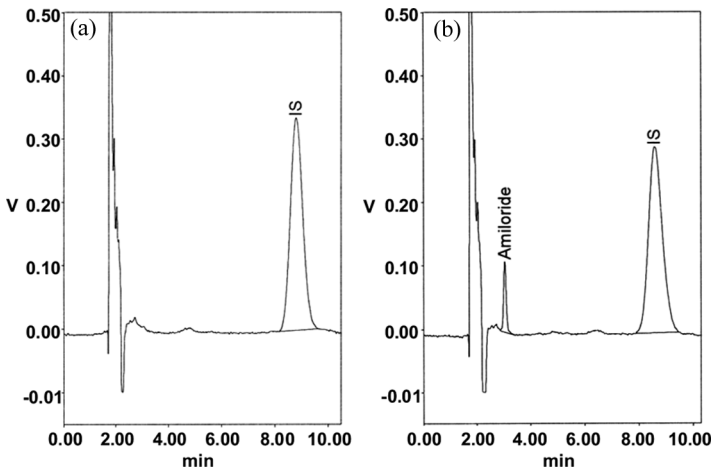

Figure 3. Representative chromatograms of (a) predose plasma and (b) plasma sample from a human subject at 24 hr after an oral administration of 10 mg amiloride (3.7 ng/mL). IS represents internal standard.

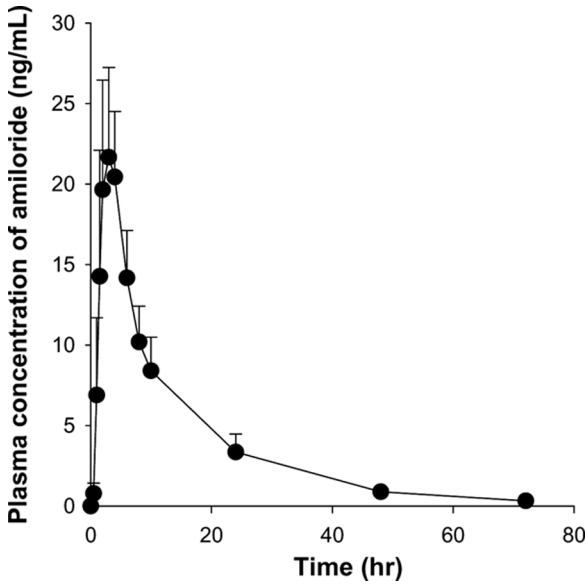

Figure 4. Mean plasma concentration versus time plots after administration of Amiloride capsule formulations to eight healthy male volunteers. The results represent the mean ± s.d. ($n = 8$).

CONCLUSION

An analytical HPLC method of amiloride from plasma samples has been developed with improving the limit of quantification (LOQ) to 0.2 ng/mL and validated. This analytical method could be successfully applied to determine amiloride in plasma of healthy volunteers who had taken amiloride tablets, and to provide data on the pharmacokinetics and bioavailability of the drug.

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REFERENCES

1. *Martindale: The Extra Pharmacopoeia*, 29th ed.; Reynolds, J.G.F., Ed.; The Pharmaceutical Press: London, 1989; 977–978 pp, 991–993 pp.

2. Brooks, S.G.; Christie, R.B.; Roche, J.; Fairhead, A.P.; Muirhead, D.; Townsend, H.A.; Shaw, H.L. Pharmacokinetics of an oral furosemide/amiloride combination tablet. Curr. Med. Res. Opin. **1984**, *9* (2), 141–146.
3. Sabanathan, K.; Castleden, C.M.; Adam, H.K.; Ryan, J.; Fitzsimons, T.J. A comparative study of the pharmacokinetics and pharmacodynamics of atenolol, hydrochlorothiazide and amiloride in normal young and elderly subjects and elderly hypertensive patients. Eur. J. Clin. Pharmacol. **1987**, *32* (1), 53–60.
4. Ismail, Z.; Triggs, E.J.; Smithurst, B.A.; Parke, W. The pharmacokinetics of amiloride-hydrochlorothiazide combination in the young and elderly. Eur. J. Clin. Pharmacol. **1989**, *37* (2), 167–171.
5. Somogyi, A.; Hewson, D.; Muirhead, M.; Bochner, F. Amiloride disposition in geriatric patients: importance of renal function. Br. J. Clin. Pharmacol. **1990**, *29* (1), 1–8.
6. Yip, M.S.; Coates, P.Z.; Tiessen, J.J. High-performance liquid chromatographic analysis of amiloride in plasma and urine. J. Chromatogr. **1984**, *307*, 343–350.
7. Forrest, G.; McInnes, G.T. Simple high-performance liquid chromatographic method for the measurement of amiloride in body fluids. J. Chromatogr. **1988**, *428*, 123–130.
8. Somogyi, A.; Keal, J.; Bochner, F. Sensitive high-performance liquid chromatographic assay for determination of amiloride in biologic fluids using an ion-pair extraction method. Ther. Drug Monit. **1988**, *10* (4), 463–468.
9. Bechgaard, E. Sensitive high-performance liquid chromatographic determination of amiloride in human plasma by direct injection with pre-column enrichment. J. Chromatogr. **1989**, *490*, 219–223.
10. Alliegro, M.A.; Dyer, K.D.; Cragoe, Jr., E.J.; Glaser, B.M.; Alliegro, M.C. High-performance liquid chromatographic method for quantitating plasma levles of amiloride and its analogues. J. Chromatogr. **1992**, *582*, 217–223.
11. Bi, H.; Cooper, S.F.; Côté, M.G. Determination and identification of amiloride in human urine by high-performance liquid chromatography and gas chromatography-mass spectrometry. J. Chromatogr. **1992**, *582* (1–2), 93–101.
12. Baer, J.E.; Jones, C.B.; Spitzer, S.A.; Russo, H.F.J. The potassium-sparing and natriuretic activity of N-amidino-3,5-diamino-6-clropyrazinecarboxamide hydrochloride dihydrate (amiloride hydrochloride). Pharmacol. Exp. Ther. **1967**, *157*, 472–485.
13. Weiss, P.; Hersey, R.M.; Dujovne, C.A.; Bianchine, J.R. The metabolism of amiloride hydrochloride in man. Clin. Pharmacol. Ther. **1969**, *10*, 401–406.
14. Reuter, K.; Knauf, H.; Mutschler, E. Florimetric determination of amiloride in human plasma using thin-layer chromatography. J. Chromatogr. **1982**, *233*, 432–436.
15. Parissi-Poulou, M.; Reizopoulou, V.; Koupparis, M.; Macheras, P. Second derivative UV spectrophotometric determination of hydrochlorothiazide and hydrochlorothiazide-amiloride combination in tablets. Int. J. Pharm. **1989**, *51* (2), 169–174.

16. Shi, R.J.Y.; Benet, L.Z.; Lin, E.T. High-performance liquid chromatographic assay of basic amine drugs in plasma and urine using a slice gel column and an aqueous mobile phase. I. Amiloride. J. Chromatogr. **1986**, *377*, 399–404.
17. Meng, Q.C.; Chen, Y.F.; Oparil, S. High-performance liquid chromatographic determination of amiloride and its analogues in rat plasma. J. Chromatogr. **1990**, *529*, 201–209.
18. Xu, D.K.; Zhou, J.H.; Yuan, Y.S. High-performance liquid chromatographic assay for amiloride in plasma and urine. J. Chromatogr. **1991**, *567*, 451–458.
19. Lee, C.; Tannock, I. Pharmacokinetic studies of amiloride and its analogs using reversed-phase high-performance liquid chromatography. J. Chromatogr. B. **1996**, *685*, 151–157.
20. Song, M.; Hang, T.; Zhao, H.; Wang, L.; Ge, P., Ma, P. Simultaneous determination of amiloride and hydrochlorothiazide in human plasma by liquid chromatography/tandem mass spectrometry with positive/negative ion-switching electrospray ionization. Rapid. Commun. Mass Spectrom. **2007**, *21*, 3427–3434.
21. Lee, Y.J.; Kim, Y.G.; Lee, M.G.; Chung, S.J.; Lee, M.H.; Shim, C.K. Analysis of bioequivalence study using log-transformed model. Yakhakhoeji. **2000**, *44* (4), 308–314.
22. Park, J.S.; Park, K.M.; Kim, C.K. Determination of triflusal in human plasma by high performance liquid chromatography with automated column switching system. J. Liq. Chromatogr. & Rel. Technol. **2000**, *23* (16), 2513–2524.
23. Smith, A.J.; Smith, R.N. Kinetics and bioavailability of two formations of amiloride in man. Br. J. Pharmacol. **1973**, *48* (4), 646–649.
24. Spahn, H.; Reuter, K.; Mutschler, E.; Gerok, W.; Knauf, H. Pharmacokinetics of amiloride in renal and hepatic disease. Eur. J. Clin. Pharmacol. **1987**, *33* (5), 493–498.
25. Jones, K.M.; Liao, E.; Hohneker, K.; Turpin, S.; Henry, M.M.; Selinger, K.; Hsyu, P.H.; Boucher, R.C.; Knowles, M.R.; Dukes, G.E. Pharmacokinetics of amiloride after inhalation and oral administration in adolescents and adults with cystic fibrosis. Pharmacotherapy **1997**, *17* (2), 263–270.
26. Jankowski, A.; Skorek-Jankowska, A.; Lamparczyk, H. Determination and pharmacokinetics of a furosemide-amiloride drug combination. J. Chromatogr. B. **1997**, *693*, 383–391.
27. Dollery, C. *Therapeutic Drugs, 2nd ed.*; Churchill Livingstone: London, 1999; A127–A129 pp.

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