ELSEVIER

Journal of Chromatography A, 797 (1998) 271–281

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatography methods for the separation and quantitation of spironolactone and its degradation products in aqueous formulations and of its metabolites in rat serum

A.M. Kaukonen[a,*], P. Vuorela[b], H. Vuorela[b], J.-P. Mannermaa[a]

[a]*Division of Pharmaceutical Technology, Department of Pharmacy, Biocenter 2, P.O. Box 56, University of Helsinki, FIN-00014 Helsinki, Finland*

[b]*Division of Pharmacognosy, Department of Pharmacy, Biocenter 2, P.O. Box 56, University of Helsinki, FIN-00014 Helsinki, Finland*

Abstract

HPLC assays have been developed for the determination of spironolactone and its degradation products 7α-thiospirolactone and canrenone in aqueous solutions of β-cyclodextrins and for the determination of spironolactone and its metabolites 7α-thiospirolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone in rat serum samples. Both methods were well suited for their respective applications, i.e., studying the stability of spironolactone in liquid formulations of β-cyclodextrins and the oral absorption of spironolactone in rats. The HPLC method developed for spironolactone and its metabolites in rat serum requires very small volumes of serum making it possible to take several blood samples over a period of time. © 1998 Elsevier Science B.V.

Keywords: Mobile phase composition; Stability studies; Spironolactone; Spirolactones; Canrenone

1. Introduction

Spironolactone, a specific aldosterone antagonist, is used as a potassium sparing diuretic in premature infants to reduce lung congestion [1]. In neonates oral medication is given through a nasogastric tube making liquid formulations preferable. As spironolactone is commercially available only in tablet form a number of suspension formulations compounded from tablets [2–5], as well as a clear liquid formulation prepared from pure spironolactone [6] have been reported. The problems presented by the poor water-solubility of spironolactone, 30 μg/ml, have often been solved by using high osmolality syrups as suspending agents [2,3,5] or high amounts of cosolvents [6], both approaches being undesirable for neonatal patients [7]. Water-soluble derivatives of β-cyclodextrin (β-CD) are therefore being considered as an alternative for the solubilization of spironolactone. In previous studies on spironolactone stability in pharmaceutical preparations, either solids or liquids, stability indicating analytical methods have been developed taking into account the degradation products canrenone [3,4,8–10] and canrenoic acid [8,9]. A new stability indicating method for the quantitation of spironolactone in aqueous β-CD solutions was needed, as previously reported nuclear magnetic resonance (NMR) studies indicated the possibility of spironolactone degradation through deacetylation in the presence of β-CD [11].

In oral absorption studies in man, serum concentrations of spironolactone or its metabolites have been followed up to 24 [12,13], 48 [14] or even 60 h [15] postdose. The sulphur-containing metabolite of

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01167-9

spironolactone, 7α-thiomethylspirolactone, has been found to be the main active metabolite in man [12] as well as in guinea pigs [16,17] seconded by the non-sulphur metabolite canrenone. Unchanged spironolactone has been detected up to 8 h after dosing in humans and it is plausible that spironolactone itself is partly responsible for its activity [12]. In guinea pigs 7α-thiomethylspirolactone, 7α-thiospirolactone and canrenone have been determined in plasma samples after intraperitoneal dosing of spironolactone, 7α-thiomethylspirolactone exhibiting higher concentrations than canrenone at 4 h postdose [17]. Previously developed methods for the determination of plasma [13,14,16,17] or serum [15,18] concentrations of spironolactone and its metabolites involved extraction procedures using sample volumes of 1 ml. Oral absorption studies of spironolactone in rats may require sampling periods similar to that in man and sample volumes are, therefore, expected to be very small. This requires a simple procedure of sample preparation without liquid–liquid or solid-phase extraction. Serum was chosen as sample material to reduce protein load as no reasons for the preference of plasma over serum have been presented [13–18].

The aim of this study was to develop high-performance liquid chromatography (HPLC) assays for the determination of spironolactone and its degradation products 7α-thiospirolactone and canrenone in aqueous solutions of β-CDs and for the determination of spironolactone and its metabolites 7α-thiospirolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone in rat serum samples.

2. Experimental

2.1. Chemicals

Methanol (MeOH), acetonitrile (MeCN) and tetrahydrofuran (THF) of HPLC-grade (Rathburn, Walkerburn, UK) were used for preparation of samples and chromatography. Deionized water was purified by a Milli-Q Plus system (Millipore, Bedford, MA, USA). Spironolactone, 7α-thiospirolactone, 7α-thiomethylspirolactone and 6β-hydroxy-7α-thiomethylspirolactone were kindly provided by Searle (Skokie, IL, USA) and canrenone by Searle (Morpeth, UK) (Fig. 1). The internal standards (I.S.s) testosterone and methyltestosterone were purchased from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA), respectively. Heptakis 2,6-di-*O*-methyl-β-cyclodextrin [degree of substitution (DS) 14] (DM-β-CD) was purchased from Sigma and 2-hydroxypropyl-β-cyclodextrin (molar substitution 0.44; DS 3.1) (Encapsin HPB) (HP-β-CD) was kindly donated by Janssen Biotech (Beerse, Belgium).

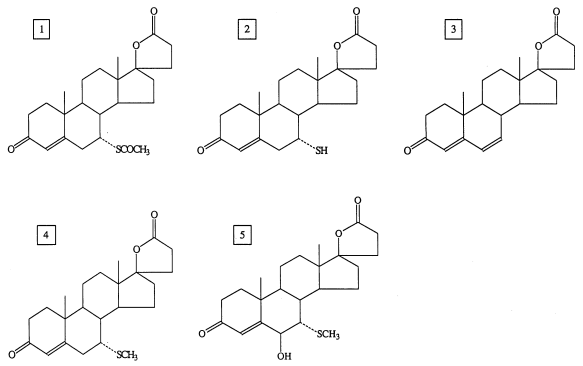

Fig. 1. Molecular structures of spironolactone (1), 7α-thiospirolactone (2), canrenone (3), 7α-thiomethylspirolactone (4) and 6β-hydroxy-7α-thiomethylspirolactone (5).

2.2. Animals and treatment

Adult male Wistar rats weighing 400–500 g were used. Rats were deprived of food for about 20–24 h before drug administration or collection of blood for blank serum. Food was provided 4 h after drug administration, while tap water was freely available during the whole time. During fasting the rats were kept in cages with wide screen bottoms to prevent coprophagy. The intraarterial catheter (polyethylene; PE-50; O.D. 0.96 mm) needed for the experiment was inserted in the carotid artery under anaesthesia (i.p. injections of xylazine 15 mg/kg and ketamine 80 mg/kg) the day before the experiment (minimum 20 h of recovery) [19]. The heparinized catheters were passed under the skin and exteriorized at the back of the neck. Blood for blank serum was withdrawn into sample containers for serum collection (Microvette CB 1000 S, Sahrstedt, Nümbrecht, Germany), centrifuged at 6000 *g* for 2 min and

stored at −20°C. A maximum of 5 ml was collected from individual rats. After that the rats were immediately killed with carbon dioxide.

A solution containing 5 mg/ml of spironolactone prepared in aqueous 48 m*M* DM-β-CD solution was administered orally by gavage at a dose of 5 mg/100 g body mass. Blood samples (0.1 ml/100 g body mass) were withdrawn before dosing (control) and at 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10, 12 and 20 h postdose and treated as above. Rats were killed as above after the last sample was collected.

2.3. Mobile phase optimization

2.3.1. Apparatus

The HPLC system consisted of a Waters 600E multisolvent delivery system, an in-line degasser, autosampler 717, programmable 991 photodiode array detection (DAD) system (Millipore) coupled to a personal computer (PC) and Waters 5200 printer plotter (Millipore).

2.3.2. Chromatographic conditions

Reversed-phase (RP) columns were used at ambient temperature; Nova-Pak C_{18}, C_8 and Phenyl steel cartridge column (Waters), 150×3.9 mm, particle size 4 μm, mean pore diameter 60 Å; LichroCart steel cartridge column packed with Spherisorb ODS-2, 250×4 mm, particle size 5 μm. The solvent rate was 1.0 ml/min and detection was effected at 254 nm. The solvent peak was treated as the dead volume. Stock solutions and samples were prepared in pure acetonitrile to ensure the stability of compounds [14].

2.3.3. The PRISMA optimization program

A computer assisted program [20] developed for the PRISMA method [21] was used in the optimization of the mobile phase. The program works under MATLAB and uses desirability functions to find the optimal selectivity with the PRISMA model.

2.4. Finalization and validation of methods

2.4.1. Apparatus

The HPLC equipment consisted of a Waters 510 solvent delivery system, in-line degasser, 717 Plus autosampler and a 490 programmable multiple-wavelength UV–Vis detection system. Data and system management was handled by a Millenium 2010 chromatography manager.

2.4.2. Chromatographic conditions

2.4.2.1. Degradation products

A Nova-Pak Phenyl steel cartridge column (Waters) (150×3.9 mm, particle size 4 μm, mean pore diameter 60 Å) with an integrated precolumn (Sentry guard column, 4 μm, 20 mm×3.9 mm, Waters) was used at ambient temperature. The solvent rate was 1.0 ml/min with detection carried out at two wavelengths: 250 nm for spironolactone and 7α-thiospirolactone and 280 nm for canrenone. A mobile phase consisting of 21.5% THF in water was used in calibration and method validation.

2.4.2.2. Metabolites

A LichroCart steel cartridge column packed with Spherisorb ODS-2 (5 μm, 250×4 mm) was used with an integrated precolumn (Spherisorb, 4×4 mm) at ambient temperature. The solvent rate was 1.0 ml/min with detection carried out at two wavelengths: 238 nm for spironolactone, 7α-thiospirolactone, 7α-thiomethylspirolactone and 6β-hydroxy-7α-thiomethylspirolactone and 280 nm for canrenone. A mobile phase consisting of 67% MeOH in water was used in calibration and method validation.

2.4.3. Calibration

2.4.3.1. Degradation products

Stock solutions were prepared in pure acetonitrile to ensure the stability of compounds [14]. Final dilutions of standards were prepared in 50% MeCN in water with four replicates of six concentrations ($n=24$). Testosterone was used as I.S. at 2.0 μg/ml. Aliquots were placed in autosampler vials and 30-μl samples injected.

2.4.3.2. Metabolites

Stock solutions and dilutions of standards were prepared in pure acetonitrile with four replicates of six concentrations ($n=24$). Methyltestosterone was used as I.S. at 0.5 μg/ml. Samples were prepared by adding 100 μl of standard solution to a 100 μl of blank rat serum. The samples were vortexed for 15 s

before centrifugation (FP-510, Labsystem, Finland) at 5000 rpm for 3 min. Aliquots of the supernatant were placed in autosampler vials and 100-μl samples injected.

2.4.4. Recovery

2.4.4.1. Degradation products

A 4% solution of DM-β-CD was prepared and spironolactone, 7α-thiospirolactone and canrenone were added. Samples were prepared by diluting 100 μl of DM-β-CD solution with pure MeCN to half the final volume and making up the desired volume with water. Unspiked samples ($n=3$) and samples spiked ($n=2$) with standard solutions at 3–4 concentration levels were determined. Estimates of the true concentration were obtained from the intercepts of the linear regression lines of determined concentration vs. spiked concentration. Recovery was calculated as percent of determined against estimated concentration.

2.4.4.2. Metabolites

Two rats were administered spironolactone and the experiments were aborted at 3.5 and 6 h postdose by collecting a blood sample of 4 ml. Unspiked samples ($n=2$ or 3) were determined by adding 100 μl of I.S. solution and spiked samples ($n=2$) by adding 100 μl of standard solutions. Results were treated as above to obtain recoveries.

3. Results and discussion

3.1. Mobile phase optimization

3.1.1. Spironolactone and degradation products

The HPLC method development for the separation of spironolactone and its degradation products 7α-thiospirolactone and canrenone (Fig. 1) was started with mobile phase optimization. THF, MeCN and MeOH were selected as possible organic components of the mobile phase. Water was used for the adjustment of solvent strength (S_T) and a C_{18} RP column (Nova-Pak) as the stationary phase. Methyltestosterone was included as potential I.S. [6]. Compositions of water and THF, MECN or MeOH producing runtimes around 10–15 min were used in the preliminary runs. In 35% (v/v) MECN in water and 60% MeOH in water spironolactone and 7α-thiospirolactone coeluted, while 25% THF in water produced coeluting peaks of 7α-thiospirolactone and methyltestosterone. Each organic solvent also produced a different retention order of the compounds.

The PRISMA model [20] was now used in the mobile phase optimization to explore the potential of binary or tertiary mixtures of these organic solvents to produce the desired separation. Altogether thirteen tertiary mixtures of THF, MECN and MeOH were used as mobile phase at $S_T=1.2$ for samples containing spironolactone, 7α-thiospirolactone, canrenone and methyltestosterone. The retention time and the width at half height of the compounds at each mobile phase composition and the dead volume were entered into the PRISMA program [21]. The program suggested one optimum area of selectivity near the MeOH corner of the solvent diagram, with the point of highest desirability at $P_S=0.2:0.8:9$ (Fig. 2). However, baseline separation of 7α-thiospirolactone and spironolactone was not obtained when the mobile phase (0.5% THF, 3% MeCN and 41.6% MeOH in water) representing the suggested selectivity point was used (Fig. 2).

As tertiary mixtures of THF, MECN and MeOH failed to produce the desired separation, work was continued without the use of I.S. In the preliminary runs with 25% THF in water (Nova-Pak C_{18}) 7α-thiospirolactone and methyltestosterone coeluted as one peak when all compounds were present in the sample. Mixtures of THF in water were now explored without methyltestosterone and baseline separation was obtained with 24% THF in water when a LichroCart column packed with Spherisorb ODS-2 was used. This gave the last eluting compound spironolactone a retention time of 61.8 min. In order to reduce the run time other RP materials (C_8 and phenyl) were tried. A shorter run time (45 min) was obtained with 21% THF in water using a Nova-Pak Phenyl column.

3.1.2. Spironolactone and metabolites

Screening of retention behaviour of spironolactone and its metabolites was started with mobile phases consisting of 35% MeCN in water, 27% THF in water and 70% MeOH in water using a LichroCart Spherisorb ODS-2 column. Samples containing

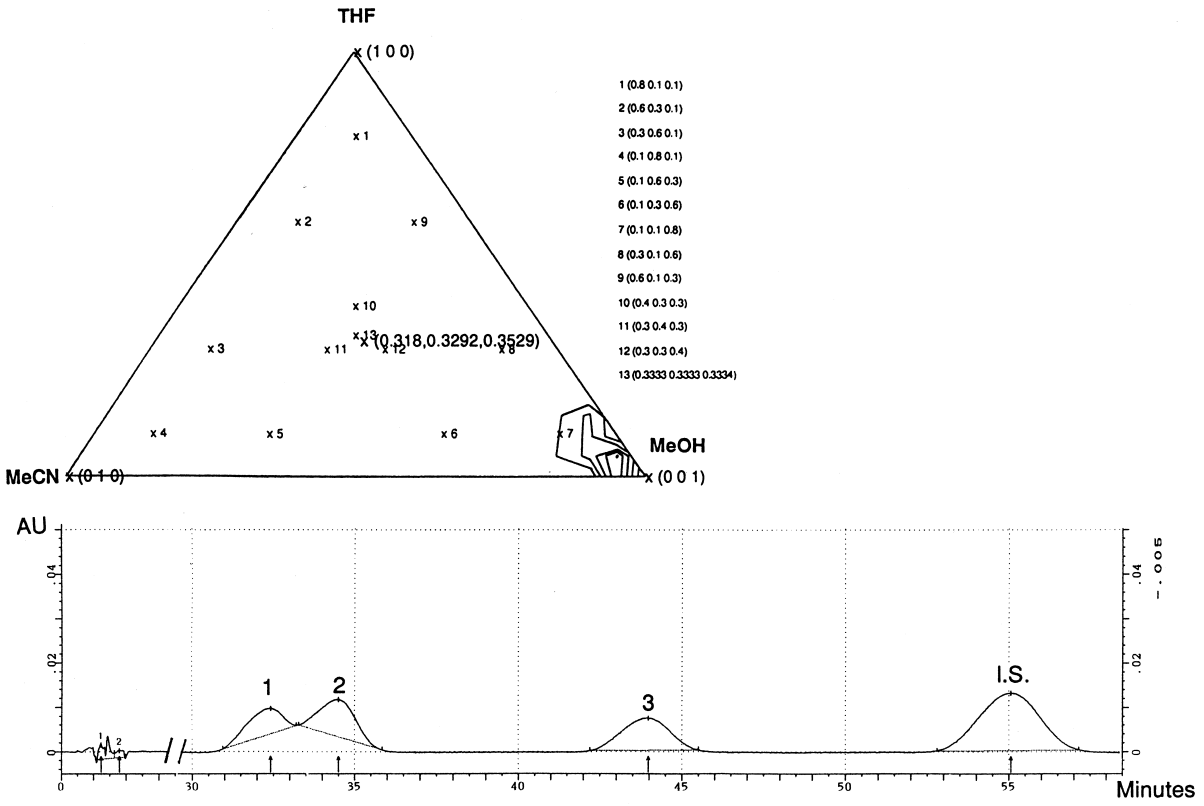

Fig. 2. Overall desirability for the resolution of spironolactone and its degradation products based on the experimental data determined at 13 selectivity points (P_S) and S_T adjusted to 1.2. The chromatogram was obtained using the mobile-phase at P_S=0.2:0.8:9 suggested by the PRISMA program and a Nova-Pak C_{18} column. Spironolactone (1); 7α-thiospirolactone (2); canrenone (3); methyltestosterone (I.S.).

spironolactone and the metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone were used (Fig. 1). 7α-Thiomethylspirolactone and canrenone coeluted in the mobile phases containing MeCN and THF, whereas four somewhat overlapping peaks could be observed in the run with MeOH. At this point MeOH was chosen as organic mobile phase component for further work.

3.2. Finalization and validation of methods

3.2.1. Spironolactone and degradation products

Work was continued with a Nova-Pak Phenyl column with an integrated precolumn giving a total column length of 170 mm. Samples with low concentrations of 7α-thiospirolactone and canrenone (0.5 μg/ml) and high concentrations of spironolactone (30 μg/ml) were used to mimic the situation in samples of the stability study. To increase sensitivity detection was carried out using two channels at wavelengths of maximum absorption for respective compounds; 238 nm for spironolactone and 7α-thiospirolactone and 280 nm for canrenone. At 238 nm the lower absorptivity of MECN used as sample solvent versus that of the mobile phase (21.5% THF in water) caused a negative baseline drift just prior to the elution of 7α-thiospirolactone. In order to improve the baseline different sample solvents and analytical wavelengths were explored. At 250 nm 0.5 μg/ml of 7α-thiospirolactone could be determined using 50% MeCN in water as sample solvent. This sample solvent also improved the peak shapes giving retention times of canrenone, 7α-thiospirolactone and spironolactone of 31.0, 35.3 and 40.0 min, respectively (Fig. 3). Several compounds of steroidal

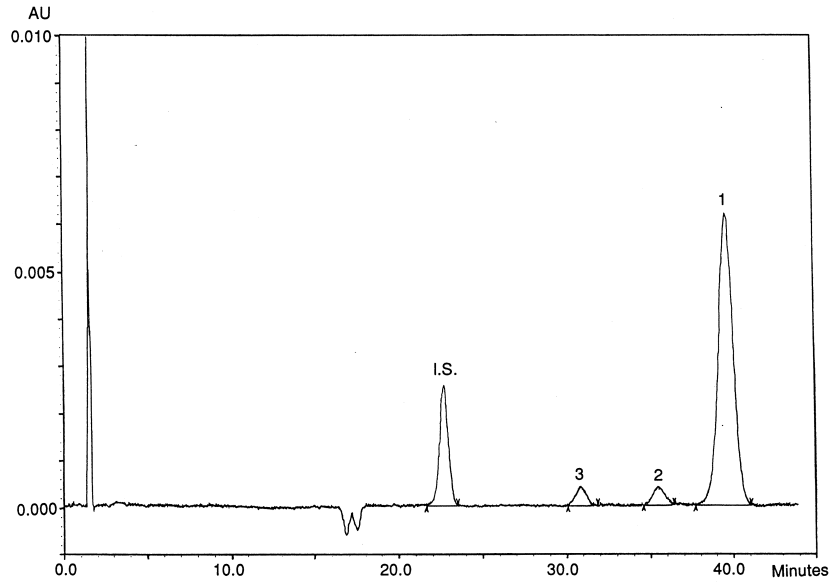

Fig. 3. Chromatogram of sample in 50% MeCN in water containing (1) spironolactone (5.7 μg/ml), (2) 7α-thiospirolactone (0.7 μg/ml), (3) canrenone (0.9 μg/ml) and testosterone (I.S.) at 250 nm.

structure were tested as potential I.S.s and testosterone with $t_R = 22.6$ min was chosen.

Calibration plots for spironolactone, 7α-thiospirolactone and canrenone were obtained in the ranges 5.0–30.0, 0.5–4.0 and 0.5–4.0 μg/ml, respectively (Table 1). Compound peak areas or compound/I.S. peak area ratios versus concentration was used in the analysis of linear regression. Testosterone determined at 250 nm was used as I.S. for canrenone as testosterone was not detected at 280 nm. The standards were prepared with the highest concentrations of spironolactone combined with the lowest concentrations of 7α-thiospirolactone and canrenone. Samples were prepared in batches and the long run time of the method meant that some of the samples were prepared 6–8 h before analysis. Spironolactone and canrenone yielded excellent linear correlation ($R^2 = 0.999$) with and without the use of I.S. (Table 1). With 7α-thiospirolactone a better linear correlation was obtained when samples were prepared on-line ($R^2 = 0.996$) than when they were prepared as a batch ($R^2 = 0.988$) (Table 2). On-line preparation was, therefore, used henceforth. Repeatibilities (R.S.D.) of the assay were obtained

Table 1
Linear regression parameters obtained from the calibration curves of spironolactone and its degradation products 7α-thiospirolactone and canrenone

Compound	Concentration range (μg/ml)	λ (nm)	Slope (mean±S.E.)	Intercept (mean±S.E.)	R^2
With internal standard					
Spironolactone	5.0–30.0	250	0.734±0.004	−0.083±0.067	0.999
7α-Thiospirolactone	0.5–4.0	250	0.403±0.009	−0.103±0.022	0.988
Canrenone	0.5–4.0	280	1.298±0.009	0.107±0.021	0.999
Without internal standard					
Spironolactone	5.0–30.0	250	66 790±356	7944±6194	0.999
7α-Thiospirolactone	0.5–4.0	250	38 381±960	108 24±2233	0.986
Canrenone	0.5–4.0	280	123 760±637	5510±1483	0.999

Table 2
Linear regression parameters obtained from the calibration curves of 7α-thiospirolactone from samples prepared as a batch or on-line using internal standard (testosterone 2.0 μg/ml)

Compound	Concentration range (μg/ml)	Slope (mean±S.E.)	Intercept (mean±S.E.)	R^2
Batch preparation				
7α-Thiospirolactone	0.5–4.0	0.403±0.009	−0.103±0.022	0.988
On-line preparation				
7α-Thiospirolactone	0.5–4.0	0.427±0.006	−0.068±0.013	0.996

from six replicate runs of individual dilutions. R.S.D.s for spironolactone, 7α-thiospirolactone and canrenone at highest concentrations were below 1.5% with or without I.S. At the lowest concentrations R.S.D.s were in the range 1.7–7.5% (Table 3). Accuracy was calculated as overall mean differences from nominal concentrations and ranged between −0.7% and 1.6% with I.S. Without I.S. the respective range was 2.5–5.0%.

The assay was developed to study spironolactone stability in solutions of DM-β-CD, HP-β-CD and sulphobutyl ether β-CD. A solution of DM-β-CD was used to determine recovery of the assay (Table 6), as NMR studies had revealed that spironolactone was stable in the presence of this CD [22]. On-line preparation of samples was used and results were processed using calibration plots with I.S. for spironolactone and 7α-thiospirolactone. Results on canrenone were processed without I.S. Mean recoveries of all compounds were in the range 93.1–98.6% (Table 6). Spironolactone stability in 22 m*M* HP-β-CD solution at 22°C was studied using the developed HPLC method and the obtained semilogarithmic plots on spironolactone and 7α-thiospirolactone concentrations vs. time are presented in Fig. 4. As spironolactone degradation in the presence of HP-β-CD indeed was found to produce 7α-thiospirolactone, it is likely that none of the previously developed methods [3,4,8–10] could have been applicable. This was supported by the results in the mobile phase optimization study, which pointed out the difficulty of simultaneously separating

Table 3
Accuracy and repeatability of the assay for spironolactone and its degradation products 7α-thiospirolactone and canrenone

Compound	*n*	Concentration (μg/ml)	With internal standard		Without internal standard	
			Determined (μg/ml)[a]	R.S.D. (%)	Determined (μg/ml)[a]	R.S.D. (%)
Spironolactone	6	30.0	29.9±0.2	0.7	30.3±0.1	0.3
	6	22.5	22.5±0.2	0.9	23.4±0.3	1.3
	6	5.0	5.0±0.1	1.7	5.0±0.1	1.9
Accuracy (%)[a]	18		99.3±1.5		102.7±1.7	
7α-Thiospirolactone	6	0.5	0.54±0.01	2.3	0.57±0.01	2.8
	6	4.0	3.8±0.1	1.4	3.9±0.1	1.5
Accuracy (%)[a]	12		101.6±6.0		105.0±9.3	
Canrenone	6	0.5	0.50±0.04	7.2	0.52±0.04	7.5
	6	1.0	0.97±0.02	2.0	1.01±0.03	2.7
	6	4.0	4.04±0.04	1.1	4.08±0.02	0.4
Accuracy (%)[a]	18		99.4±4.5		102.5±4.8	

Analyzed samples prepared on-line.
[a] Mean±S.D.

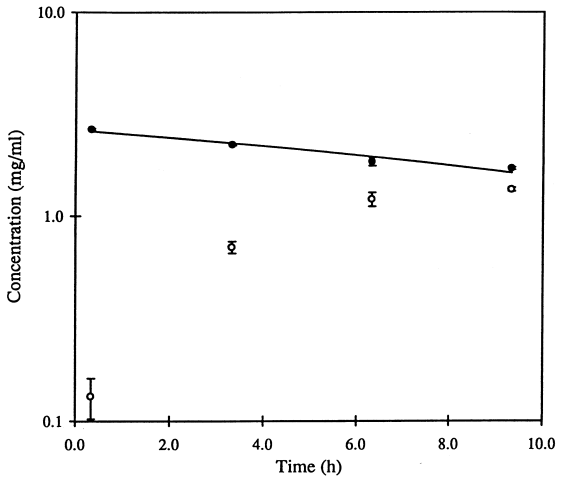

Fig. 4. Concentration (mean±S.D.; $n=4$) vs. time profiles of spironolactone (●) and 7α-thiospirolactone (○) in 22 m*M* HP-β-CD solution stored at 22°C plotted on a semilogarithmic scale; initial spironolactone concentration 3 mg/ml.

spironolactone, 7α-thiospirolactone and canrenone under isocratic conditions. As the separation of spironolactone and 7α-thiospirolactone was crucial for the intended stability study the developed method was considered suitable despite the rather long run time.

3.2.2. Spironolactone and metabolites

Work was started by exploring preparation of serum samples. Different volume ratios of MeCN vs. serum were used to find the ratio producing sufficient precipitation of serum proteins. A clear supernatant was produced when 1:1 serum and MeCN was used and the samples were vortexed for 15 s prior to centrifugation at 5000 rpm for 3 min. Samples were prepared by adding 100-μl of solution containing spironolactone and all metabolites to 100 μl blank serum. A LichroCart Spherisorb ODS-2 column with an integrated precolumn was used and detection was carried out at 238 and 280 nm. Separation of 6β-hydroxy-7α-thiomethylspirolactone, spironolactone, 7α-thiospirolactone, 7α-thiomethylspirolactone and canrenone was obtained in 11 min with a mobile phase consisting of 67% MeOH in water (Fig. 5B). 6β-Hydroxy-7α-thiomethylspirolactone was eluted first ($t_R=5.05$ min) and appeared in the downward slope of the sample peak. Analysis of blank serum showed that one or two endogenous peaks eluted in

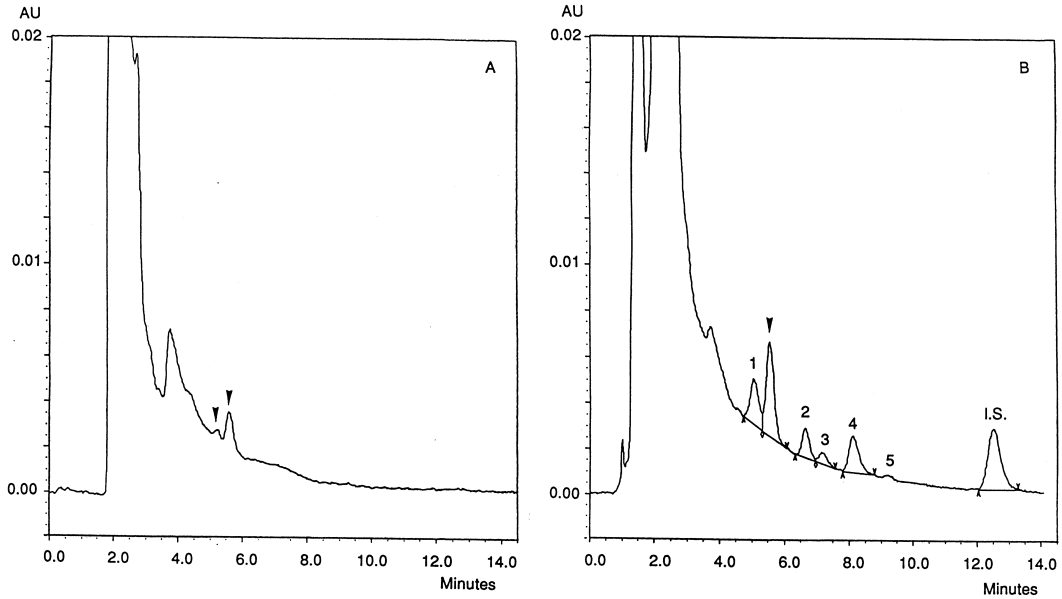

Fig. 5. Chromatogram of (A) blank rat serum and (B) blank serum spiked with (1) 6β-hydroxy-7α-thiomethylspirolactone (200 ng/ml), (2) spironolactone (152.7 ng/ml), (3) 7α-thiospirolactone (148.2 ng/ml), (4) 7α-thiomethylspirolactone (300 ng/ml) and (5) canrenone (100 ng/ml) at 238 nm. Methyltestosterone as I.S. at 0.5 μg/ml. Arrows indicate endogenous peaks.

its vicinity (Fig. 5A). Spironolactone (t_R=6.61 min) and 7α-thiospirolactone (t_R=7.17 min) had overlapping peaks but baseline separation was obtained between 7α-thiospirolactone and 7α-thiomethylspirolactone (t_R=8.02 min). The slight overlap of 7α-thiomethylspirolactone and canrenone (t_R=9.12 min) peaks caused little disturbance at 238 nm due to the low absorptivity of canrenone at this wavelength. The overall separation of the compounds was considered acceptable as quantitation of the main active metabolites 7α-thiomethylspirolactone and canrenone was held most important. Methyltestosterone (t_R=12.65 min) was chosen as I.S. giving a runtime of 15 min for the assay.

Calibration plots for spironolactone, 7α-thiospirolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone were obtained in the ranges 102–509, 99–494, 100–2000, 150–500 and 50–1200 ng/ml, respectively (Table 4). Linear regression analysis of compound/I.S. peak height ratios versus concentration in serum gave correlation coefficients (R^2) of 0.997, 0.987, 0.999, 0.894 and 0.999, respectively. The interfering endogenous peaks can explain the poor linear correlation of 6β-hydroxy-7α-thiomethylspirolactone in comparison with the other compounds. Mean accuracies for the assay ranged between −11.8% and 13.4% for all compounds with 6β-hydroxy-7α-thiomethylspirolactone and 7α-thiospirolactone at respective extreme (Table 5). R.S.D.s were below 12%. The samples collected at 3.5 and 6 h postdose contained 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone. Recoveries determined from these samples were in the range 93.1–117.3% (Table 6).

The serum concentration–time profile for spironolactone metabolites determined in rat serum after oral administration of spironolactone is presented in Fig. 6. These results demonstrate the suitability of the method for its intended use in the assessment of oral bioavailability of spironolactone from β-CD solutions. A small sample volume presents a prerequisite for following the serum concentrations of spironolactone metabolites over a longer period of time in rats. Sample volumes as small as 50 μl have been utilized in a method where spironolactone and canrenone have been determined in urine [23]. Collection of urine samples from rats would, however, present a problem. 7α-Thiomethylspirolactone has been found to be the principal metabolite in serum/plasma both in man [12] and guinea pig [17], making serum samples desirable for the assessment of spironolactone absorption. 7α-Thiospirolactone has not been reported as plasma metabolite of spironolactone in man and in some of the methods developed for studying human subjects 7α-thiospirolactone has been omitted [13,18]. The determination of 7α-thiospirolactone from rat serum was considered important as 7α-thiospirolactone has been identified in guinea pig plasma [17]. The used HPLC method separated spironolactone, 7α-thiospirolactone and 7α-thiomethylspirolactone but required the sacrifice of the animal at the time of sampling to obtain the necessary volume of plasma for liquid extraction. Furthermore, a methanol–water gradient was employed giving a total run time of 40 min. Intravenous administration of spironolactone has shown that 7α-thiospirolactone also can be determined as serum metabolite in rats [24].

4. Conclusions

A stability indicating isocratic HPLC method for spironolactone in aqueous solutions of β-CDs has

Table 4
Linear regression parameters obtained from the calibration curves of spironolactone and its metabolites in rat serum

Compound	λ (nm)	Concentration range (ng/ml)	Slope (mean±S.E.)	Intercept (mean±S.E.)	R^2
Spironolactone	238	102–509	1.682±0.020	0.013±0.006	0.997
7α-Thiospirolactone	238	99–494	0.603±0.015	0.022±0.005	0.987
7α-Thiomethylspirolactone	238	100–2000	1.113±0.006	−0.014±0.006	0.999
Hydroxy-7α-thiomethylspirolactone	238	150–500	1.237±0.093	0.162±0.030	0.894
Canrenone	280	50–1200	1.851±0.011	−0.015±0.006	0.999

Table 5
Accuracy and repeatability of the assay for spironolactone and its metabolites

Compound	*n*	Concentration (ng/ml)	Determined (ng/ml)[a]	R.S.D. (%)
Spironolactone	5	153	144±5	3.5
	6	509	492±11	2.3
Accuracy (%)[a]	11		95.4±2.8	
7α-Thiospirolactone	5	148	151±18	12.0
	6	494	614±16	2.6
Accuracy (%)[a]	11		113.4±14.1	
7α-Thiomethylspirolactone	5	300	285±5	1.7
	6	2000	1993±23	1.2
Accuracy (%)[a]	11		95.7±1.5	
Hydroxy-7α-thiomethylspirolactone	6	500	446±24	5.5
Accuracy (%)[a]	6		88.2±4.9	
Canrenone	6	100	99±6	6.1
	6	1200	1183±20	1.7
Accuracy (%)[a]	12		98.9±4.3	

[a] Mean±S.D.

been developed. It provides a simple, selective and reliable method for the separation and quantitation of spironolactone and its degradation products 7α-thiospirolactone and canrenone. The isocratic HPLC method developed for spironolactone and its metabolites in rat serum was found well suited for the intended purpose. The method requires very small volumes of serum and separates spironolactone and

Table 6
Recovery of the assay for spironolactone and its degradation products in aqueous DM-β-CD solution or its metabolites in rat serum samples

Compound	*n*	Determined (μg/ml)[a]	*n*	Estimated (μg/ml)[a]	Recovery (%)[a]
DM-β-CD solution					
Spironolactone	3	15.0±0.1	3	15.2±0.1	98.6±0.8
	3	5.67±0.07	3	6.1±0.1	93.1±1.0
7α-Thiospirolactone	3	0.65±0.02	3	0.69±0.02	94.6±2.1
Canrenone	3	0.93±0.01	3	0.93±0.03	98.3±3.6
Rat serum[b]					
7α-Thiomethylspirolactone	3	896±18	3	956±84	94.1±7.9
	2	1386±61	3	1404±79	98.9±5.6
Canrenone	3	557±15	3	548±62	102.6±11.4
	2	451±15	3	444±33	101.9±7.9
Hydroxy-7α-thiomethylspirolactone	3	257±10	4	220±18	117.3±9.6

[a] Mean±S.D.
[b] Rat serum ''Determined'' and ''Estimated'' values are in (ng/ml).

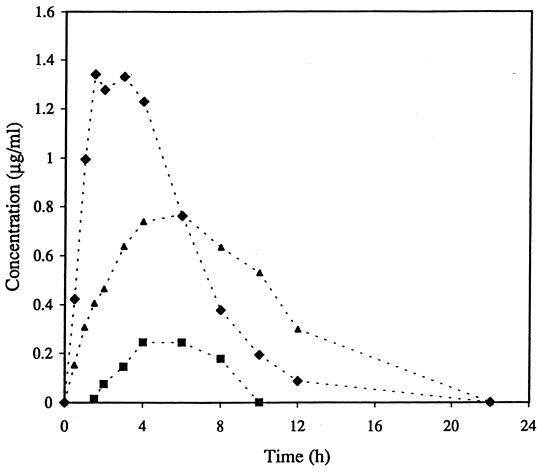

Fig. 6. Serum concentration–time profile of spironolactone metabolites determined in rat after oral administration of 5 mg/100 g body mass of spironolactone in DM-β-CD solution. (♦) 7α-Thiomethylspirolactone; (▲) canrenone; (■) 6β-hydroxy-7α-thiomethylspirolactone.

all metabolites of interest providing a reliable method for the quantitation of the main active metabolites 7α-thiomethylspirolactone and canrenone.

Acknowledgements

We gratefully acknowledge Janssen, Biotech, Beerse, Belgium for the donation of hydroxypropyl-β-cyclodextrins and Searle and Co. for supplying spironolactone and degradation products and metabolites. The Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmacy, University of Helsinki, is acknowledged for the use of their HPLC equipment during the finalization and validation of methods. A.M.K. acknowledges partial financial support from the Jenny and Antti Wihuri Foundation.

References

[1] S.A. Atkinson, J.K. Shah, C. McGee, B.T. Steele, J. Pediatr. 113 (1988) 540.
[2] V.D. Gupta, C.W. Gibbs, A.G. Ghanekar, Am. J. Hosp. Pharm. 35 (1978) 1382.
[3] L.K. Mathur, A. Wickman, Am. J. Hosp. Pharm. 46 (1989) 2040.
[4] G.M. Peterson, M.F. Meaney, C.A. Reid, G.R. Taylor, Aust. J. Hosp. Pharm. 19 (1989) 344.
[5] M.C. Nahata, R.S. Morosco, T.F. Hipple, Ann. Pharmacother. 27 (1993) 1198.
[6] Y. Pramar, V.D. Gupta, C. Bethea, J. Clin. Pharm. Ther. 17 (1992) 245.
[7] R. Leff, R. Roberts, Am. J. Hosp. Pharm. 44 (1987) 865.
[8] F. De Croo, W. Van den Bossche, P. De Moerloose, J. Chromatogr. 325 (1985) 395.
[9] F. De Croo, W. Van den Bossche, P. De Moerloose, J. Chromatogr. 329 (1985) 422.
[10] Y. Pramar, V.D. Gupta, T. Zerai, Drug Dev. Ind. Pharm. 17 (1991) 747.
[11] D. Wouessidjewe, A. Crassous, D. Duchêne, A. Coleman, N. Rysanek, G. Tsoucaris, B. Perly, F. Djedaini, Carbohydr. Res. 192 (1989) 313.
[12] H.W.P.M. Overdiek, W.A.J.J. Hermens, F.W.H.M. Merkus, Clin. Pharmacol. Ther. 38 (1985) 469.
[13] A. Jankowski, A. Skorek-Jankowska, H. Lamparczyk, J. Pharm. Biomed. Anal. 14 (1996) 1359.
[14] F. Varin, T.M. Tu, F. Benoît, J.-P. Villeneuve, Y. Théorêt, J. Chromatogr. 574 (1992) 57.
[15] H. Zhang, J.T. Stewart, J. Pharm. Biomed. Appl. 11 (1993) 1341.
[16] J.H. Sherry, J.P. O'Donnell, H.D. Colby, J. Chromatogr. 374 (1986) 183.
[17] L.E. Los, A.B. Coddington, H.G. Ramjit, H.D. Colby, Drug Metab. Dispos. 21 (1993) 1086.
[18] J.W.P.M. Overdiek, W.A.J.J. Hermens, F.W.H.M. Merkus, J. Chromatogr. 341 (1985) 279.
[19] H. Lennernäs, C.-G. Regårdh, Pharm. Res. 10 (1993) 130.
[20] K. Outinen, H. Vuorela, R. Hiltunen, Eur. J. Pharm. Sci. 4 (1996) 199.
[21] Sz. Nyiredi, B. Meier, C.A.J. Erdelmeier, O. Sticher, J. High Resolut. Chromatogr., Chromatogr. Commun. 8 (1985) 186.
[22] A.M. Kaukonen, I. Kilpeläinen, J.-P. Mannermaa, Int. J. Pharm. 159 (1997) 159.
[23] R. Herráez-Hernández, E. Soriano-Vega, P. Campíns-Falcó, J. Chromatogr. B 658 (1994) 303.
[24] A.M. Kaukonen, H. Lennernäs, J.-P. Mannermaa, J. Pharm. Pharmacol. submitted for publication.