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Development and validation of an HPLC method for the determination of spironolactone and its metabolites in paediatric plasma samples☆

J.M. Sandall, J.S. Millership*, P.S. Collier, J.C. McElnay

Clinical and Practice Research Group, School of Pharmacy, Queen's University Belfast, Medical Biology Centre, 97, Lisburn Road, Belfast BT9 7BL, UK

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Abstract

An HPLC method has been developed and validated for the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in paediatric plasma samples. The method utilises 200 μl of plasma and sample preparation involves protein precipitation followed by Solid Phase Extraction (SPE). Determination of standard curves of peak height ratio (PHR) against concentration was performed by weighted least squares linear regression using a weighting factor of 1/concentration^2. The developed method was found to be linear over concentration ranges of 30–1000 ng/ml for spironolactone and 25–1000 ng/ml for 7α-thiomethylspirolactone and canrenone. The lower limit of quantification for spironolactone, 7α-thiomethylspirolactone and canrenone were calculated as 28, 20 and 25 ng/ml, respectively. The method was shown to be applicable to the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in paediatric plasma samples and also plasma from healthy human volunteers.

Keywords: Spironolactone; 7α-Thiomethylspirolactone; Canrenone; HPLC; Pharmacokinetics; Children

1. Introduction

Spironolactone (**1**, Fig. 1) is a potassium-sparing diuretic and is marketed as a competitive aldosterone antagonist and is indicated for the treatment of oedema and ascites in cirrhosis of the liver, malignant ascites, nephrotic syndrome, congestive heart failure and primary hyperaldosteronism [1]. The use of spironolactone in infants and children was first reported in 1964 when administration of the drug to three infants produced a moderate diuresis [2]. Since then spironolactone has been widely utilized in the management of congestive heart failure associated with congenital heart disease [3–5], bronchopulmonary dysplasia or chronic lung disease [5–9] and paediatric ascites [10,11]. Although frequently prescribed, there is a lack of published research documenting the safety and efficacy of spironolactone in the paediatric population. In a recent review, Brion et al. [12] concluded that despite widespread use of spironolactone in preterm infants with chronic lung disease, the clinical outcomes of such treatment had been evaluated in surprisingly few infants in randomized clinical trials. The need for further investigation in order to clarify the role of spironolactone in the treatment of chronic lung disease was highlighted [12]. Subsequently in 2003, the National Institutes of Health in the USA included spironolactone as one of twelve drugs on the first "List of Drugs for Which Pediatric Studies Are Needed" [13]. This list is compiled annually under directive from the Best Pharmaceuticals for Children Act (2002) [14], to prioritize off-patent drugs for which paediatric studies are most urgently needed in order to ensure their safety and efficacy. In addition to the paucity of safety and efficacy data, the pharmacokinetic (PK) properties of spironolactone have not been reported in infants and children nor has the metabolism. In adults, spironolactone is rapidly absorbed from the gastrointestinal tract [15,16]. Plasma protein binding is extensive (88–98%) and occurs to both human serum albumin and α_1-acid glycoprotein [16,17]. Spironolactone is rapidly and extensively metabolized to a large number of metabolites [15,16,18,19]. The major metabolic pathways of spironolactone result in the formation of 7α-thiomethylspirolactone (**2**, Fig. 1) and canrenone (**3**, Fig. 1). It is probable that both metabolites contribute to the antimineralocorticoid effects of

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* Corresponding author. Tel.: +44 2891 272025.
E-mail address: j.millership@qub.ac.uk (J.S. Millership).

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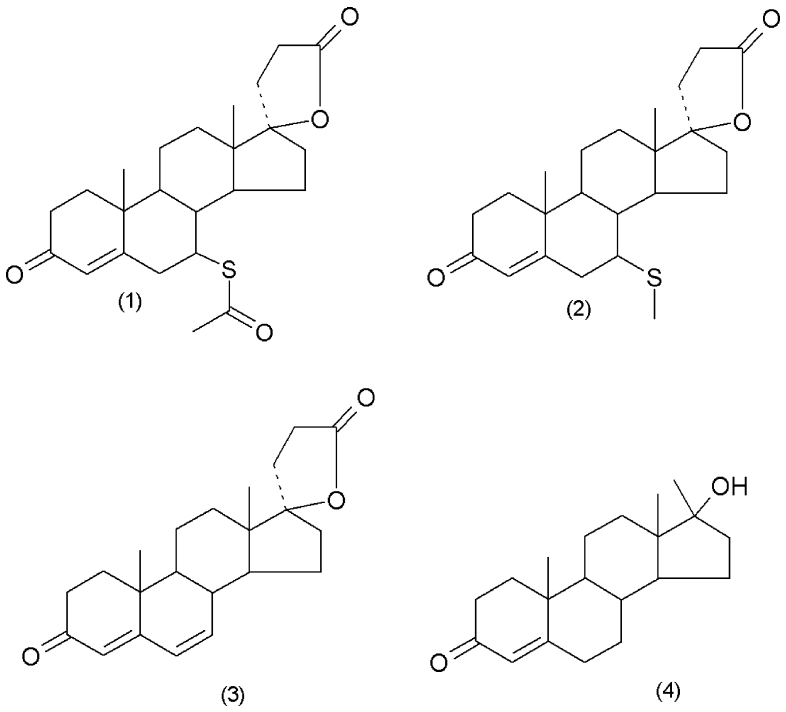

Fig. 1. Structures of spironolactone (**1**), 7α-thiomethylspirolactone (**2**), canrenone (**3**) and 17α-methyltestosterone (**4**) (IS).

spironolactone, however, the higher affinity for renal aldosterone receptors coupled with the increased plasma concentrations of 7α-thiomethylspirolactone, indicate that it is the more important of the two [20].

As part of an ongoing research programme we are currently employing population PK analysis of sparse data to gain knowledge of the PK profiles of several drugs which are routinely used in an off-label or unlicensed manner for the treatment of children. One of the drugs under investigation is spironolactone. This work required the availability of an appropriate assay method for the simultaneous determination of spironolactone and the active metabolites 7α-thiomethylspirolactone (**2**) and canrenone (**3**) in plasma. An essential prerequisite of the assay was the ability to accurately quantify low concentrations of all three compounds from small volume samples, as paediatric plasma samples are often 200 μl or lower. In addition, sample preparation should be quick and efficient, facilitating use of the assay for routine analysis. Numerous HPLC methods [21–33] have been developed for the determination of spironolactone and/or its metabolites in plasma or serum. A number of these methods are unsuitable for the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in paediatric plasma samples either because not all three compounds were determined or the plasma sample size was at least 1 ml. Only one method, that of Kaukonen et al. [33], utilised a small sample size (100 μl of rat serum) and determined all three components. The present paper describes the development of a HPLC method for the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in paediatric plasma samples utilising a method based, in part, on that reported by Kaukonen et al. [33] but which also involved the development of a SPE clean up procedure.

2. Experimental

2.1. Materials

All reagents were of analytical grade except where otherwise stated. Spironolactone and 17α-methyltestosterone (**4**, Fig. 1), used as the internal standard (IS), were purchased from Sigma (Poole, UK). 7α-Thiomethylspirolactone was synthesized at the School of Pharmacy, Queen's University Belfast according to a technical report produced by Searle Research and Development [34] and its identity confirmed by NMR spectroscopy and melting point determination. Canrenone was kindly donated by Pharmacia (Morpeth, UK). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Loughborough, UK). All water was HPLC grade and obtained using a Millipore Direct-Q™ 5 Water System (Millipore, Watford, UK; supplied through AGB Scientific Apparatus Ltd., Belfast). Filtration of HPLC mobile phases was performed using Sartorius membrane filters 0.45 μm obtained from Sartorius (Epsom, UK). Oasis® HLB solid phase extraction cartridges (1 cc/30 mg) were purchased from Waters (Dublin, Ireland). Plasma was kindly donated by the Northern Ireland Blood Transfusion Service and stored at −20 °C until required.

2.2. Instrumentation

Solid phase extraction was carried out using a Zymark RapidTrace™ SPE Workstation (Zymark, Runcorn, UK; supplied through AGB Scientific Apparatus Ltd., Belfast). The HPLC system consisted of a Waters 1525 Binary HPLC pump, a Waters 717 plus autosampler, a Waters 2487 Dual λ Absorbance

detector and a Jones Chromatography column block heater. Data were acquired and integrated using Breeze™ software (Waters, Dublin, Ireland). Solvent evaporation was performed using a Zymark TurboVap® LV Evaporator Workstation (Zymark, Runcorn, UK; supplied through AGB Scientific Apparatus Ltd., Belfast).

2.3. Chromatographic conditions

Chromatographic separation was achieved using reverse phase chromatography with isocratic elution. The method employed was a modification of that described by Kaukonen et al. [33]. Separation was performed using a Waters Symmetry® C_{18} (150 mm × 4.6 mm, 5 μm) analytical column fitted with a Waters Symmetry® C_{18} (20 mm × 3.9 mm, 5 μm) sentry guard column, both maintained at 28 °C. UV detection was carried out at two wavelengths, 238 nm for spironolactone, 7α-thiomethylspirolactone and 17α-methyltestosterone, and 280 nm for canrenone. The mobile phase, consisting of methanol:water (60:40, v/v), was filtered and degassed through a 0.45 μm filter and pumped at a flow rate of 1 ml/min. The injection volume was 40 μl.

2.4. Construction of standard curves for spironolactone, 7α-thiomethylspirolactone and canrenone in plasma

Master stock and working standard solutions were prepared in methanol and stored in the dark at 4 °C until required. Working standard solutions were prepared at seven concentrations for spironolactone, 7α-thiomethylspirolactone and canrenone ($n = 21$), and at 20 ng/ml for 17α-methyltestosterone (IS). Plasma standards were prepared by adding 2.5 μl of each of the appropriate spironolactone, thiomethylspirolactone, canrenone and IS working standard solutions to 200 μl of blank plasma, resulting in concentrations of 30, 50, 100, 250, 500, 750 and 1000 ng/ml for spironolactone, 25, 50, 100, 250, 500, 750 and 1000 ng/ml for 7α-thiomethylspirolactone and canrenone, and 250 ng/ml for the IS. The plasma standards were then extracted according to the procedure detailed below.

2.5. Collection and preparation of healthy volunteer and patient plasma samples

Collection of paediatric plasma samples at the two study site hospitals (The Royal Belfast Hospital for Sick Children and Alder Hey Hospital, Liverpool) was approved by the local ethical committees. Healthy volunteer and patient blood samples were collected in 5 ml EDTA sample tubes and centrifuged at 1800 × *g* for 10 min to separate the plasma component. The plasma fraction was transferred to a clean sample tube and stored at −20 °C until analysis. At the time of analysis, samples were prepared by adding 2.5 μl of the IS working standard solution (20 ng/ml) and 7.5 μl of methanol to 200 μl of the healthy volunteer or patient plasma samples. The samples were then extracted according to the procedure detailed below.

2.6. Extraction procedure

Plasma samples were prepared as detailed above and pretreated for SPE by the addition of 200 μl acetonitrile to precipitate plasma proteins. After centrifugation at 9000 × *g* for 5 min, 370 μl of the supernatant was transferred to a disposable borosilicate glass culture tube and diluted to 1 ml by the addition of 630 μl of HPLC grade water. The samples were then extracted using Oasis® HLB cartridges (1 cc/30 mg) on a Zymark RapidTrace™ SPE Workstation according to the procedure detailed in Fig. 2. The eluents were collected in disposable borosilicate glass culture tubes and evaporated to dryness at 37 °C under nitrogen using a Zymark TurboVap® LV Evaporator Workstation. The residues were reconstituted in 150 μl of mobile phase and 40 μl aliquots were injected in duplicate onto the HPLC system.

2.7. Assay validation

The following parameters were evaluated for the validation of the developed method:

- selectivity
- linearity
- accuracy
- limit of detection and lower limit of quantification
- recovery
- stability

2.7.1. Selectivity

The selectivity of the developed method was determined by analysing blank plasma samples, from six sources, for interferences when compared with plasma samples spiked with the three

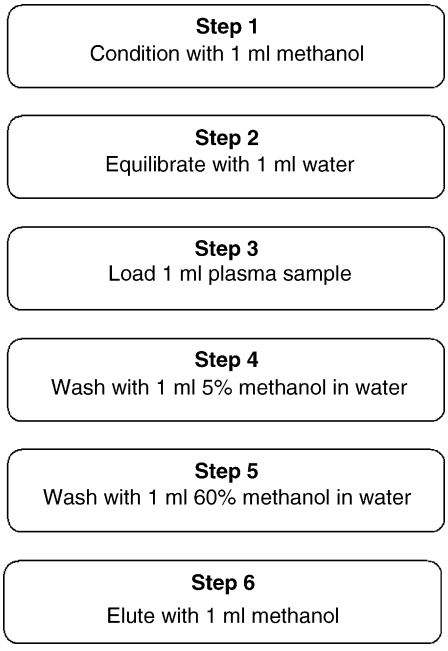

Fig. 2. Solid phase extraction procedure for spironolactone and metabolites.

analytes and IS, and plasma samples obtained from a healthy volunteer after administration of spironolactone. Potential interference from concomitant medication taken by paediatric patients was investigated by analysing mobile phase or plasma which had been spiked with the appropriate drug.

2.7.2. Linearity

The linearity of the developed method was evaluated by preparing standard curves for the three analytes on six consecutive days. Plots of peak height ratio (response) against analyte concentration were constructed. The behaviour of the response variance across the calibration range was assessed graphically by plots of studentized residual against log concentration [35] and statistically, by comparing the variances at the highest and lowest concentration using a one-sided *F*-test [36].

2.7.3. Accuracy

The bias and precision of the developed method was determined from the analysis of plasma samples spiked with the three analytes at three concentrations representing the low, medium and high portions of the standard curves (40, 400 and 950 ng/ml for spironolactone and 30, 400 and 950 ng/ml for 7α-thiomethylspirolactone and canrenone). Intra-day bias and precision were calculated on a single day using five replicates at each concentration level. Inter-day bias and precision were calculated using five replicates at each concentration level over five consecutive days. Bias and precision should not exceed 15% [37].

2.7.4. Limit of detection and lower limit of quantification

Standard curves were prepared for the three analytes in the concentration range of the LOD (7.5–20 ng/ml) and the following equation was used to calculate the LOD for each compound [38]:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

where σ is the standard deviation of the response (estimated from the standard deviation of *y*-intercepts of regression lines) and *S* is the slope of the standard curve.

Standard curves were prepared for the three analytes in the concentration range of the LLOQ (15–50 ng/ml) and the following equation was used to calculate the LLOQ for each [37]:

$$\mathrm{LLOQ} = \frac{10\sigma}{S}$$

where σ is the standard deviation of the response (estimated from the standard deviation of *y*-intercepts of regression lines) and *S* is the slope of the standard curve. The LLOQ was validated by determining the accuracy with which samples prepared at the LLOQ were quantified. Intra-day bias and precision were calculated on a single day using five replicates. Inter-day bias and precision were calculated using three replicates over 3 consecutive days. At the LLOQ, bias and precision should not exceed 20% [37].

2.7.5. Recovery

The efficiency of the extraction procedure was determined by the analysis of plasma samples spiked with the three analytes at three concentrations (30, 400 and 950 ng/ml). Six replicates at each concentration level were extracted and analysed, and the responses compared with those of non extracted standards which represent 100% recovery. The recovery of the IS was evaluated at 250 ng/ml.

2.7.6. Stability

The stability of spironolactone, 7α-thiomethylspirolactone and canrenone in plasma was investigated in samples obtained from a dosed volunteer. Three plasma samples were analysed on two occasions, five months apart, after storage at −20 °C. For each sample, the ratio of the two concentrations of each analyte was calculated. The mean ratio and standard deviation for each analyte was then determined.

2.8. Data analysis

All data were analysed using Microsoft® Excel 2002 (Microsoft Corporation, USA) with the exception of the determination of standard curves which was performed by weighted least squares linear regression analysis using SPSS® 11.5 for Windows® (SPSS Inc., USA).

3. Results and discussion

3.1. Extraction and chromatography

Initial studies based on the HPLC method of Kaukonen et al. [33] enabled separation of spironolactone, 7α-thiomethylspirolactone, canrenone and IS within a run time of 22 min. Dual wavelength UV detection was employed to optimize sensitivity by enabling detection at both 238 nm (λ_{max} for spironolactone and 7α-thiomethylspirolactone and 17α-methyltestosterone) and 280 nm (λ_{max} for canrenone). 17α-Methyltestosterone, as used in the method of Kaukonen et al. [33] was selected as the internal standard (IS) since it did not interfere with any of the analytes. When initially investigating the extraction of plasma samples according to the method described by Kaukonen et al. [33] problems arose. This method utilized acetonitrile induced protein precipitation as the sole means of sample preparation, followed by injection of the supernatant onto the HPLC system. Unfortunately the lowest plasma concentration at which this method permitted detection of the three analytes was 25 ng/ml. In addition, after only 30 injections, the system pressure had risen from 18.6 MPa to over 30 MPa, resulting in system shutdown. This increase in pressure was found to be due to inadequate clean-up of the plasma samples which resulted in the retention of unremoved endogenous plasma components on the guard column and subsequent blockage of that column. Due to these difficulties a combined method involving protein precipitation and SPE was developed to clean up the samples prior to HPLC.

Plasma samples containing spironolactone, 7α-thiomethylspirolactone and canrenone were pretreated by the addition

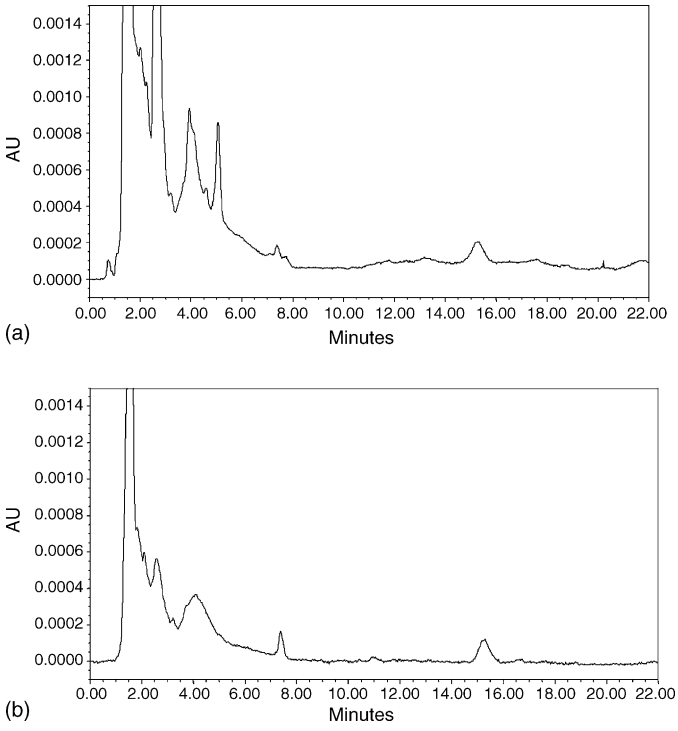

Fig. 3. Chromatograms of a blank plasma sample extracted by SPE, at (a) 238 nm and (b) 280 nm.

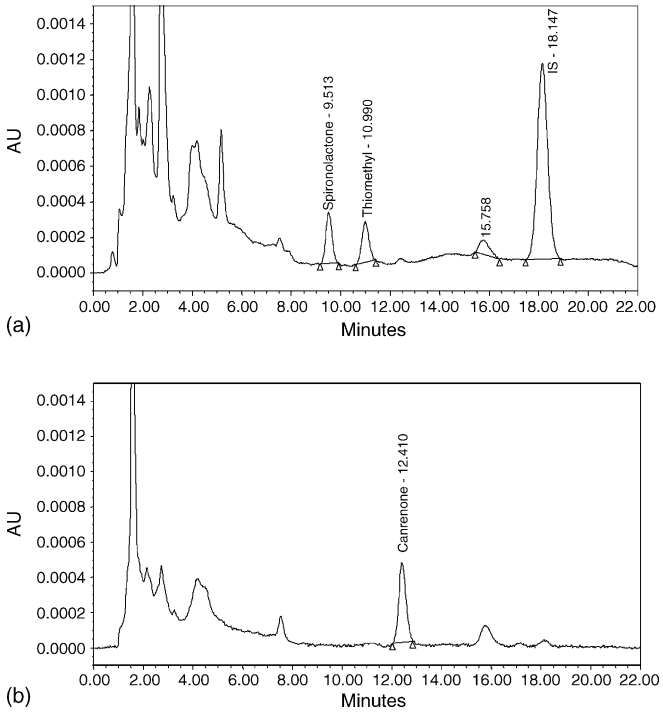

Fig. 4. Chromatograms of a blank plasma sample spiked with spironolactone (50 ng/ml), 7α-thiomethylspirolactone (50 ng/ml), canrenone (50 ng/ml) and IS (250 ng/ml) and extracted by SPE, at (a) 238 nm and (b) 280 nm.

of acetonitrile to precipitate plasma proteins, centrifuged and extracted utilizing Oasis® HLB cartridges (1 cc/30 mg). The SPE procedure was optimised by varying the concentration of methanol used in the washing solvent in order to ensure optimal removal of endogenous components. HPLC analysis of washing and elution fractions revealed that removal of endogenous plasma interferents was enhanced by increasing the methanol content of the washing solvent. Partial elution of all four compounds occurred with a 80% methanol wash. Rinsing with 60% methanol was shown to provide maximum removal of the later eluting, more non-polar, plasma interferents without causing elution of the analytes. However, it was also observed that a 5% methanol wash was most effective at removing those aqueous soluble, plasma components which were unretained by the HPLC method, as indicated by the higher absorbance of the plasma front. Optimal removal of endogenous plasma components without elution of the analytes was therefore obtained by means of a SPE procedure employing two consecutive washing steps, with 5 and 60% methanol in water, respectively. The four compounds were eluted from the cartridge using 100% methanol. A blank aqueous sample, extracted using the developed procedure, showed no interferences from the cartridge material.

The chromatograms obtained from the analysis of a blank plasma sample extracted by SPE are presented in Fig. 3. Fig. 4 shows that the three analytes and IS were well resolved from any endogenous components in spiked blank plasma. The retention times for spironolactone, 7α-thiomethylspirolactone, canrenone and IS were 9.5, 11.0, 12.4 and 18.1 min, respectively.

3.2. Assay validation

3.2.1. Selectivity

Chromatograms from extracted samples of blank plasma and plasma spiked with the three analytes were presented previously in Figs. 3 and 4. No endogenous matrix components eluted at the retention times of the peaks of interest and resolution for all peaks was >2. In addition, no interferences were observed in extracted plasma samples from a healthy volunteer receiving spironoactone (data not presented). Fig. 5 shows the concomitant medication taken by 10% or more of the paediatric patients from whom plasma samples have been collected. Several of these drugs do not contain a chromophore, e.g. chloral hydrate, netilmicin and colistin, and therefore interference with the current assay is highly unlikely. Many are known to be more hydrophilic than either spironolactone or its metabolites, e.g. furosemide, paracetamol, codeine and digoxin, and were therefore expected to have much shorter retention times than spironolactone. This was confirmed by analyses of mobile phase samples spiked with the appropriate drug. Midazolam and promethazine are lipophilic drugs which, on investigation of spiked mobile phase samples, were found to elute after spironolactone. HPLC analysis of blank plasma spiked separately with both drugs and extracted via SPE, showed no interference with any of the peaks of interest. No concomitant medication was found to interfere with the current assay. The developed method was therefore found to be selective for spironolactone, 7α-thiomethylspirolactone and canrenone in the presence of endogenous matrix components and concomitant medication.

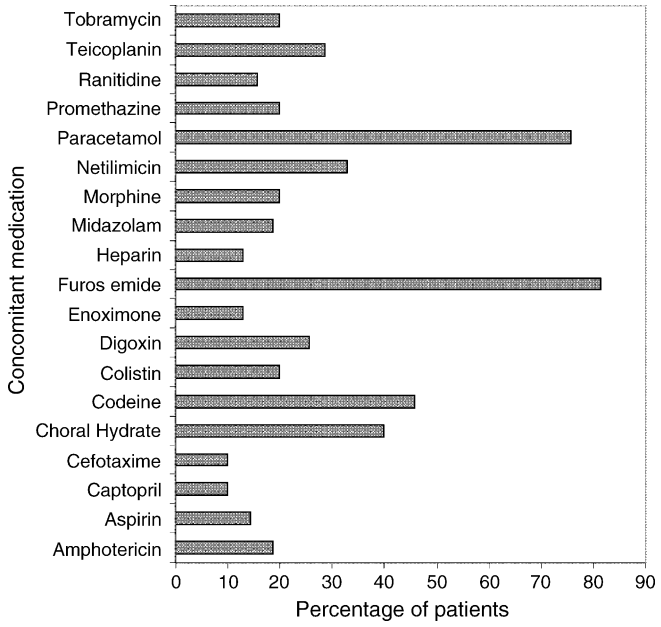

Fig. 5. Concomitant medication taken by paediatric patients ($n = 70$).

3.2.2. Linearity

The standard curves (PHR versus concentration) ranged from 30–1000 ng/ml for spironolactone and 25–1000 ng/ml for both 7α-thiomethylspirolactone and canrenone. The variances of the PHR at the highest and lowest concentration were compared for each analyte over 6 days using a one-sided F-test. The results indicated that for all three analytes, the variance at the highest concentration was significantly larger (p value <0.001) than that at the lowest concentration. Plots of studentized residual against log concentration also revealed an increase in the variance of PHR with increasing concentration, shown by the larger spread of residuals at higher concentrations. Since the three analytes displayed heteroscedasticity over the concentration range, the standard curves were obtained using weighted least squares linear regression. Graphs of the standard deviation of PHR against concentration showed that for all three analytes, standard deviation was directly proportional to concentration indicating that weighting by 1/concentration^2 would be most appropriate. This was confirmed on examination of the residual plots obtained from analyses using weighting factors of 1/concentration and 1/concentration^2. The best improvement was observed when data were weighted by 1/concentration^2. Determination of standard curves of PHR against concentration was therefore performed by weighted least squares linear regression using a weighting factor of 1/concentration^2. The slope, y-intercept and correlation coefficient of the regression line was calculated for each analyte on all 6 days, the developed method was found to be linear over concentration ranges of 30–1000 ng/ml for spironolactone and 25–1000 ng/ml for 7α-thiomethylspirolactone and canrenone.

3.2.3. Accuracy

The intra- and inter-day bias for the developed assay ranged between −9.4 and 1.7%, −6.3 and 1.2% and −11.3 and 5.0% for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively. The corresponding intra- and inter-day precision (CV) ranged between 3.2 and 8.7%, 3.8 and 9.2% and 2.2 and 7.8% for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively (Table 1). All results for bias and precision were within the acceptable limits. The developed method was therefore found to be accurate over the studied concentration ranges.

3.2.4. Limit of detection and lower limit of quantification

The LOD for spironolactone, 7α-thiomethylspirolactone and canrenone were calculated as 9, 9 and 6 ng/ml, respectively. The guideline for validation of analytical procedures produced by the International Conference on Harmonization, recommends that when the LOD is determined by calculation, it may subsequently be validated by analysis of a suitable number of samples known to be near or prepared at the LOD [38]. Fig. 6 shows the chromatograms of an extracted blank plasma sample which had been spiked with spironolactone (10 ng/ml), 7α-thiomethylspirolactone (10 ng/ml) and canrenone (7.5 ng/ml). The LLOQ for spironolactone, 7α-thiomethylspirolactone and canrenone were calculated as 28, 20 and 25 ng/ml, respectively. The bias and precision with which blank plasma samples spiked with spironolactone (30 ng/ml), 7α-thiomethyl-

Table 1
The intra- and inter-day bias and precision (CV) for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively

Nominal concentration (ng/ml)	Precision						Bias		
	Spironolactone		7α-Thiomethyl-spirolactone		Canrenone		Spironolactone (% bias)	7α-Thiomethyl-spirolactone (% bias)	Canrenone (% bias)
	Mean ± S.D. (ng/ml)	CV%	Mean ± S.D. (ng/ml)	CV%	Mean ± S.D. (ng/ml)	CV%			
Intra-day ($n = 5$)									
30/40[a]	37.5 ± 2.7	7.2	28.1 ± 1.3	4.6	31.4 ± 0.7	2.2	−6.3	−6.3	4.7
400	403.1 ± 16.1	4.0	385.7 ± 18.4	4.8	373.7 ± 11.3	3.0	0.8	−3.6	−6.6
950	860.6 ± 74.9	8.7	961.1 ± 69.0	7.2	843.1 ± 49.9	5.9	−9.4	1.2	−11.3
Inter-day ($n = 5$)									
30/40[a]	37.3 ± 3.0	8.0	29.3 ± 2.7	9.2	30.0 ± 2.0	6.7	−6.8	−2.3	0
400	406.7 ± 13.0	3.2	400.2 ± 25.2	6.3	420.1 ± 32.8	7.8	1.7	0.1	5.0
950	888.1 ± 55.5	6.2	898.2 ± 34.5	3.8	907.0 ± 23.9	2.6	−6.5	−5.5	−4.5

[a] 30 ng/ml, 7α -thiomethylspirolactone and canrenone; 40 ng/ml, spironolactone.

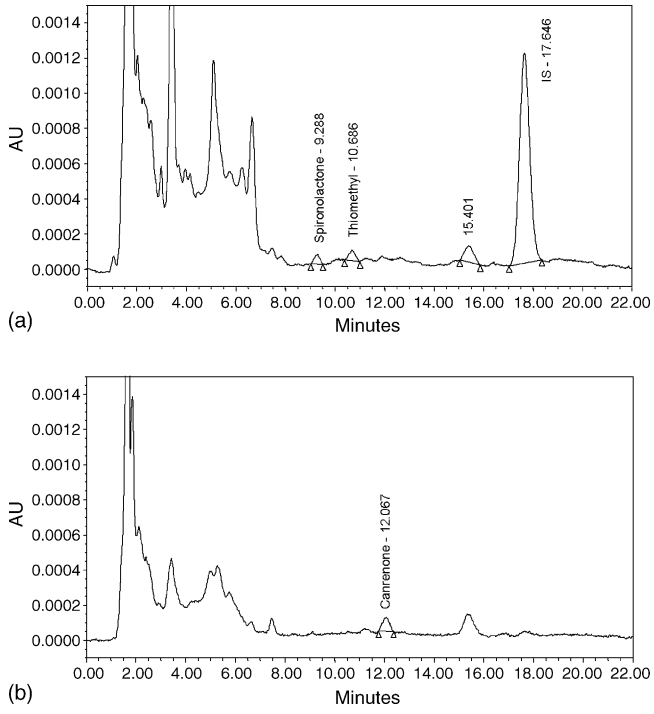

Fig. 6. Chromatograms of a blank plasma sample spiked with spironolactone (10 ng/ml), 7α-thiomethylspirolactone (10 ng/ml), canrenone (7.5 ng/ml) and IS (250 ng/ml) and extracted by SPE, at (a) 238 nm and (b) 280 nm.

spirolactone (25 ng/ml) and canrenone (25 ng/ml), were quantified and all results were within the acceptable limits (Fig. 2). The developed method was therefore validated at the LLOQ of 30, 25 and 25 ng/ml for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively (Table 2).

3.2.5. Recovery

The efficiency of the extraction procedure for the three analytes at three concentrations (30, 400 and 950 ng/ml), is presented in Table 3 Recovery ranged between 78.1 and 87.2%, 82.0 and 102.2% and 97.0 and 101.8% for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively. Recovery of the IS was 87.6% ± 4.2, at a concentration of 250 ng/ml.

3.2.6. Stability

The mean and standard deviation of the concentration ratios from the three samples were 1.02 ± 0.04, 0.98 ± 0.03 and 1.10 ± 0.06 for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively. All three analytes were therefore stable in plasma for at least five months when stored at −20 °C.

3.2.7. Analysis of healthy volunteer samples

The developed method was applied successfully for the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in plasma, after the oral administration of 100 mg spironolactone to a non-fasting healthy male volunteer. Venous blood samples were obtained immediately prior to administration of spironolactone and 15, 30, 45 min and 1, 1½, 2, 3, 4, 6 and 8 h after drug administration. The samples were collected, extracted and chromatographed as described previously. Plots of plasma concentration against time for the three analytes are displayed in Fig. 7. All three compounds were first detected 1 h after administration, however, spironolactone and canrenone were at levels below their respective LLOQ. The spironolactone and canrenone plasma concentrations 1 h post dose were determined to be 25 and 10 ng/ml, respectively, and are indicated on the graph to better illustrate the concentration-time profiles for the drugs. $C_{p_{max}}$ values of 52.6, 292.7 and

Table 2
Intra- and inter-day bias and precision for the determination of spironolactone, 7α-thiomethylspirolactone and canrenone in plasma, at their respective lower limit of quantification

Nominal concentration (ng/ml)	Precision						Bias		
	Spironolactone		7α-Thiomethyl-spirolactone		Canrenone		Spironolactone (% bias)	7α-Thiomethyl-spirolactone (% bias)	Canrenone (% bias)
	Mean ± S.D. (ng/ml)	CV%	Mean ± S.D. (ng/ml)	CV%	Mean ± S.D. (ng/ml)	CV%			
Intra-day ($n = 5$)									
25/30[a]	28.4 ± 0.6	2.1	28.5 ± 1.1	3.9	27.9 ± 0.5	1.8	−5.3	14.0	11.6
Inter-day ($n = 3$)									
25/30[a]	28.5 ± 1.8	6.3	25.5 ± 2.3	9.0	26.3 ± 1.1	4.2	−5.0	2.0	5.2

[a] 25 ng/ml, 7α-thiomethylspirolactone and canrenone; 30 ng/ml, spironolactone.

Table 3
Recovery of spironolactone, 7α-thiomethylspirolactone and canrenone from plasma following solid phase extraction

Nominal concentration (ng/ml)	Recovery (%)					
	Spironolactone		7α-Thiomethyl-spirolactone		Canrenone	
	Mean ± S.D.	CV%	Mean ± S.D.	CV%	Mean ± S.D.	CV%
30	85.1 ± 4.8	5.6	82.0 ± 3.6	4.4	97.0 ± 4.1	4.2
400	78.1 ± 3.9	5.0	88.8 ± 4.7	5.3	100.8 ± 2.0	2.0
950	87.2 ± 2.5	2.9	102.2 ± 2.7	2.6	101.8 ± 2.8	2.8

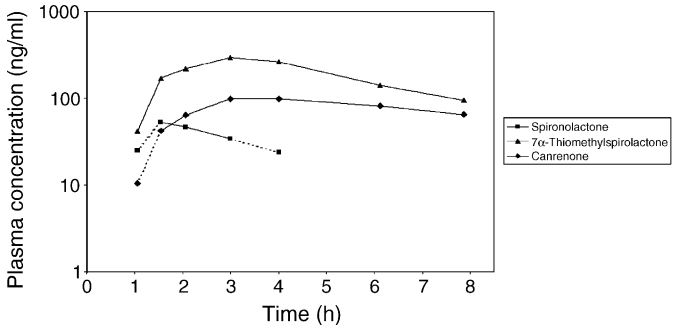

Fig. 7. Plasma concentration-time profiles for spironolactone, 7α-thiomethylspirolactone and canrenone after administration of 100 mg spironolactone to a healthy volunteer.

98.4 ng/ml were quantified after $1\frac{1}{2}$, 3 and 4 h for spironolactone, 7α-thiomethylspirolactone and canrenone, respectively. Four hours after administration, the plasma concentration of spironolactone had fallen below the LLOQ, this may indicate that a more sensitive assay for the determination of spironolactone might be appropriate. An increase in spironolactone sensitivity might allow a better insight into the absorption and metabolism of this drug. We do, however, believe that the LLOQ (for spironolactone) in this assay is as good or superior to previously reported assays and that the sensitivity of the assay for 7α-thiomethylspirolactone and canrenone, the two metabolites responsible for the diuretic effect, are appropriate for the determination of these drugs in both adult and paediatric patients. This is in contrast with 7α-thiomethylspirolactone and canrenone which were determined at levels of 94.6 and 64.8 ng/ml, respectively, 8 h after administration.

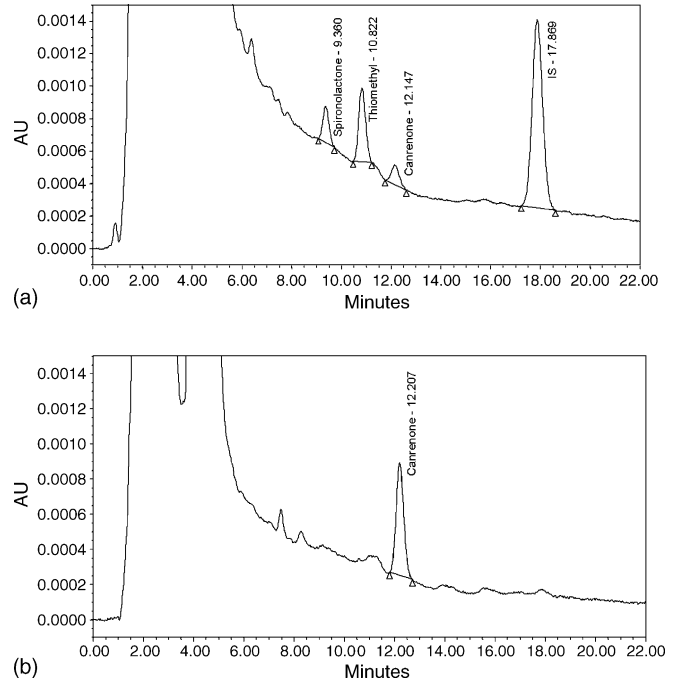

Fig. 8. Chromatograms of patient sample, spiked with IS (250 ng/ml) and extracted by SPE, at (a) 238 nm and (b) 280 nm.

3.2.8. Analysis of paediatric patient samples

The developed method was utilized for the analysis of plasma samples from four paediatric patients. In the current research programme, concentration–time profiles for investigated drugs are obtained from sparse data. Samples from patients receiving the drug therapeutically are collected at the time of routine blood sampling which occurs at random time intervals during the clinical care of the patient. To date, 152 plasma samples have been obtained from 70 patients receiving spironolactone. Samples were collected at two study sites—The Royal Belfast Hospital for Sick Children and Alder Hey Hospital, Liverpool. The chromatograms of an extracted plasma sample from one paediatric patient (female, 24 days old) who had received spironolactone (dose 4 mg) are shown in Fig. 8. The sample was taken 1.4 h after the dose and the presence of spironolactone and its active metabolites are clearly visible.

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