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High-performance liquid chromatographic determination of spironolactone and its metabolites in human biological fluids after solid-phase extraction

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic procedure to determine spironolactone and its three major metabolites in biological specimens is described. The assay involves sequential extraction on C_{18} and CN solid phases, and subsequent separation on a reversed-phase column. In plasma samples, spironolactone and its metabolites were completely separated within 8 min using an isocratic mobile phase, while in urine samples a methanol gradient was necessary to achieve a good separation within 14 min. Recoveries for all analytes were greater than 80% in plasma and 72% in urine. Linear responses were observed for all compounds in the range 6.25–400 ng/ml for plasma and 31.25–2000 ng/ml for urine. The plasma and urine methods were precise (coefficient of variation from 0.8 to 12.5%) and accurate (−12.1% to 7.4% of the nominal values) for all compounds. The assay proved to be suitable for the pharmacokinetic study of spironolactone in healthy human subjects.

INTRODUCTION

Spironolactone (SL) is a competitive inhibitor of aldosterone which has been used for almost thirty years in the therapy of essential hypertension, congestive cardiac failure and other edematous states [1–4]. Since the clearance of SL from plasma is extremely rapid, the resulting diuresis with sodium loss and potassium retention has been attributed to its metabolites. Early non-specific fluorometric assays were based on the assumption that canrenone, the dethioacetylated derivative of SL, was the major active circulating metabolite [5–7]. However, since the development of a specific high-performance liquid chromatographic (HPLC) assay for the determination of canrenone [8], many pharmacokinetic studies in humans have confirmed that canrenone levels were previously overestimated [9–12]. The presence of sulfurcontaining metabolites of SL in human plasma [13,14] as determined by specific HPLC methods [15,16] as well as their very high affinity for the aldosterone receptor [17,18] have emphasized their potential contribution to the diuretic effect of SL.

Biotransformation of SL is likely to proceed through the formation of 7α-thio-SL (I) [19], which is either methylated into 7α-thiomethyl-SL (II), desulfurized into canrenone (IV) or oxidized into a thiyl radical IX) (for review see refs. 19 and

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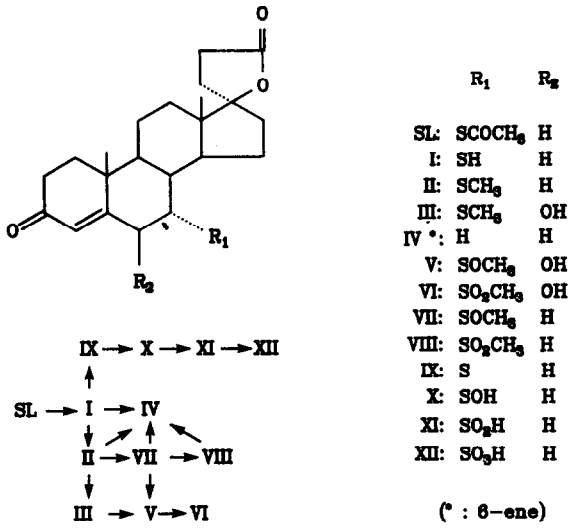

Fig. 1. Proposed pathways for the biotransformation of spironolactone (SL). Abbreviations: I = 7α-thio-SL; II = 7α-thiomethyl-SL; III = 6β-hydroxy-7α-thiomethyl-SL; IV = canrenone; V = 6β-hydroxy-7α-methylsulfinyl-SL; VI = 6β-hydroxy-7α-methylsulfonyl-SL; VII = 7α-methylsulfinyl-SL; VIII = 7α-methylsulfonyl-SL; IX = 7α-thiyl-SL; X = 7α-sulfenic-SL; XI = 7α-sulfinic-SL; XII = 7α-sulfonic-SL. Canrenone may be further hydrolysed (canrenoate), reduced (di-, tetra- and hexahydro derivatives) or hydroxylated.

20). These secondary metabolites may also undergo further metabolism as represented in Fig. 1. So far, only the parent compound and metabolites II, III and IV have been isolated in human plasma after oral administration of single or multiple doses of SL [13,14]. These HPLC analyses [15,16] were based on liquid–liquid extraction techniques which, in our hands, were unable to ensure an adequate purification of the samples without loosing some of the analytes. Although some HPLC methods claim a limit of detection between 5 and 20 ng/ml for most analytes, the limit of quantitation, as derived from their calibration curves, was 12.5 ng/ml in guinea pig plasma [16] but only 50 ng/ml in human plasma [15].

Quantification of SL and its metabolites in urine appears essential for the determination of the pharmacokinetic–pharmacodynamic relationship in patients on diuretic therapy. However, the reported techniques based on the extraction of plasma samples may not be applicable to urine. Although canrenone has been measured in urine by means of HPLC [19], there are no HPLC assays available for the determination of sulfur-containing metabolites in the urine of spironolactone-treated subjects. For these purposes, a solid-phase extraction method which is applicable to the analysis of spironolactone and its metabolites both in human plasma and urine was developed.

EXPERIMENTAL

Chemicals

Methanol, acetonitrile, hexane, ethyl acetate, dichloromethane and phosphoric acid (85%) of HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA) were used for extraction and chromatography. Deionized water was highly purified by a Milli-Q Plus system (Millipore, Bedford, MA, USA). SL, 7α-thio-SL, 7α-thiomethyl-SL, 6β-hydroxy-7α-thiomethyl-SL and canrenone were kindly provided by Searle (Chicago, IL, USA). The internal standard (I.S.), 16α,17α-epoxyprogesterone, was purchased from Sigma (St. Louis, MO, USA). All glassware was silanized by exposure to dimethyldichlorosilane-saturated atmosphere (10%, v/v in heptane) followed by methanol vapor and rinsed with diethyl ether before use.

Standard solutions

Concentrated stock solutions of spironolactone and its metabolites were prepared separately at a concentration of 1 mg/ml in acetonitrile. Working solutions in acetonitrile contained 20 or 200 μg/ml of each analyte. A stock solution of the internal standard was prepared at a concentration of 1 mg/ml in acetonitrile. The working solution contained 1 μg/ml internal standard in water.

Calibration curves

A stock of pooled human plasma containing 400 ng/ml spironolactone and its metabolites was diluted serially with drug-free plasma to give final plasma concentrations ranging from 6.25 to 400 ng/ml. A stock solution in human urine was prepared at 2 μg/ml and diluted with drug-free urine to concentrations ranging from 31.25 to 2000 ng/ml. All biological standards were stored at −20°C.

High-performance liquid chromatography

An LDC Milton Roy (Riviera Beach, FL, USA) solvent delivery system comprising two ConstaMetric III pumps, a dynamic mixer and a gradient master (Model 1601) was used. The sample was injected via a Rheodyne injector (Cotati, CA, USA) in a 100-μl loop. A Spherisorb S5 ODS1 (125 mm × 4.9 mm I.D.), singly end-capped, 5-μm column (Hichrom, Reading, UK) preceded by a 0.45-μm prefilter (SSI, State College, PA, USA) was used for the separation of spironolactone and its metabolites. The UV absorbance (Model 441, Waters Assoc., Milford, MA, USA) of column effluent was first monitored at 254 nm (for SL, metabolites II and III) and then at 280 nm (for metabolite IV). The length between the cells on both sides of the mercury lamp was kept to a minimum. The peaks at 254 and 280 nm were recorded with a C-R6A integrator (Shimadzu, Kyoto, Japan) and a BBC/Metrawatt/Goerz chart recorder (Broomfield, CO, USA), respectively.

Separation of spironolactone and its metabolites in plasma was achieved with an isocratic mobile phase consisting of methanol–water (65:35) acidified to pH 3.4 with phosphoric acid. The flow-rate was set at 2.0 ml/min. For urine samples, compounds were separated with a 55–65% 10-min concave gradient (curve 3) of methanol in water (pH 3.4), at a flow-rate of 2.0 ml/min. The chromatographic system was operated at room temperature and at a pressure between 172 and 193 bar.

Extraction procedures

Plasma. The Bond-Elut (Analytichem International, Harbor City, CA, USA) C_{18} extraction columns were activated before use by washing with 2 × 2.5 ml of methanol, followed by 2 × 2.5 ml of water. A vacuum of 50–80 kPa was applied to the manifold of the Vac-Elut chamber (Analytichem International) at each stage of the extraction procedure. Plasma (1-ml sample or standard), 100 μl of I.S. and 1 ml of water were combined in the reservoir and passed through the column. This was followed by 2 × 1.5 ml of water, and 1 ml of methanol–water (40:60). Analytes were eluted with 2 × 1 ml of acetonitrile, and the combined eluates were evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA). The residue was solubilized in 100 μl of ethyl acetate and diluted with 1.5 ml of hexane. The organic phase was transferred and passed through Bond-Elut CN columns (vacuum less than 35 kPa), which had been preactivated by washing with 2 × 2.5 ml of methanol, followed by 2 × 2.5 ml of hexane. The columns were washed with 2 ml of hexane and analytes eluted with 2 × 0.75 ml of acetonitrile. The combined eluates were evaporated as above. The residue was dissolved in 200 μl of the HPLC eluent immediately before analysis and 100 μl of the solution were injected onto the column.

Urine. Urine (1-ml sample or standard) and 1 ml of I.S. were combined in the reservoir of activated C_{18} Bond-Elut columns and passed through. The columns were washed with 1.5 ml of water, twice, followed by 1.5 ml of acetonitrile–water (35:65), twice. After elution with acetonitrile, the remaining extraction procedure was similar to that described for plasma.

Recovery

Extraction recovery of spironolactone and its metabolites from serum and urine was assessed with spiked samples at two different levels over the relevant concentration ranges. Recovery estimates for plasma (25 and 200 ng/ml) and urine (62.5 and 1000 ng/ml) were based on a comparison of the analyte/internal standard peak-height ratio obtained after the extraction of spiked samples to the peak-height ratio of a similar amount of the analytes added directly to the tube before the last evaporation. The recovery of the internal standard, which was assessed separately by comparing with the peak-height response obtained after direct on-column injection of the same amount of internal standard, was complete.

Linearity and limit of quantitation

Linear calibration plots of spironolactone and its metabolites were generated by least-square regression of the analyte/internal standard peak-height ratios against their respective concentration in plasma (6.25–400 ng/ml) or urine (31.25–2000 ng/ml). No reliance was placed onto the detection limit based on the usual signal-to-noise ratio 3:1. The minimum quantifiable level was de-

termined as the lowest concentration of standard which gave a regression-estimated value within 15% of the nominal concentration.

Precision and accuracy

Intra-assay and inter-assay variabilities were determined by replicate measures of SL and its metabolites in plasma (25 and 200 ng/ml) and urine (62.5 and 1000 ng/ml) on the day of preparation and on different days, respectively. All data were derived from the same standards used in the calibration curves. The accuracy of the method was determined by assaying, under blind conditions, ten samples of plasma and urine spiked with various concentrations of SL and its metabolites.

RESULTS AND DISCUSSION

While setting up the assay it became obvious that the stability of some analytes was affected by solvents. Sherry *et al.* [16] have previously shown that SL, when kept in methanol, was slowly converted into 7α-thio-SL. We have also noticed that 6β-hydroxy-7α-thiomethyl-SL, 7α-thio-SL and 7α-thiomethyl-SL, when kept in a dichloromethane–hexane mixture, were each degraded within minutes into one or two different products that were more polar than the parent drug (Fig. 2). A difference in the sensitivity of these metabolites to degradation was observed, the 7α-thiomethyl-SL being the most sensitive while the 7α-thio-SL was the least sensitive. The rate of degradation was found to be directly proportional to the concentration of dichloromethane in the organic mixture. This solvent-induced degradation may explain the low recovery of these sulfur-containing metabolites after dichloromethane extraction in earlier studies [5–7]. This raises some doubts as to whether the oxidation of 7α-thiomethyl-SL into 7α-thiomethyl-SL S-oxide is catalyzed by enzymes [21,22] or is a mere consequence of dichloromethane extraction. Decker *et al.* [23] have shown that the addition of *m*-chloroperbenzoic acid to SL dissolved in dichloromethane induced its degradation into sulfinic and sulfonic acid derivatives. Although the same degradation was observed with various lots and brands of HPLC

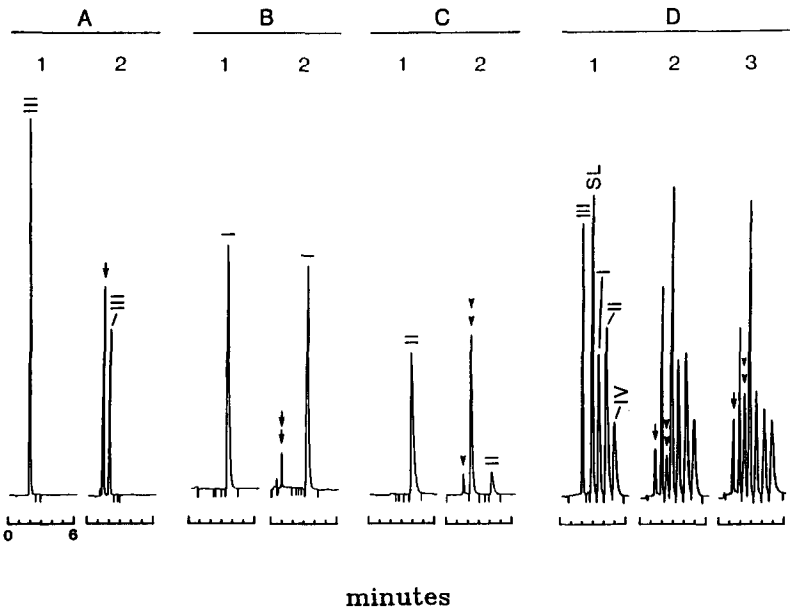

Fig. 2. Chromatograms of standards of III (A), I (B), II (C) and III, SL, I, II and IV (D). Standard solutions were injected directly onto the column (1) or added to a mixture of dichloromethane–hexane (50:50 in A, B and C; 10:90 in D) which was either immediately evaporated (2) or kept at room temperature for 30 min before evaporation (3). Evaporated samples were appropriately diluted in the mobile phase and injected in amounts comparable to those directly injected. Single arrows, double arrows and arrowheads indicate degradation products of III, I and II, respectively.

TABLE I

RECOVERIES OF SPIRONOLACTONE AND ITS METABOLITES FROM HUMAN PLASMA AND URINE AFTER TWO CONSECUTIVE SOLID-PHASE EXTRACTIONS

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)			
	SL	II	III	IV
Plasma (n = 4)				
25	80.4 ± 3.0	85.4 ± 4.2	93.7 ± 5.0	87.1 ± 5.9
200	91.3 ± 1.4	90.5 ± 3.6	91.8 ± 3.7	89.5 ± 0.7
Urine (n = 6)				
62.5	73.4 ± 6.1	76.3 ± 5.3	75.6 ± 6.1	72.2 ± 4.0
1000	82.5 ± 6.8	75.3 ± 1.6	83.8 ± 3.8	83.3 ± 3.7

dichloromethane, its extent was minimal when recently opened bottles were used. We did not observe any breakdown of sulfur-containing metabolites when they were kept in chloroform, tetrachloromethane, acetonitrile, ethyl acetate or hexane. Spironolactone and canrenone were stable when they were kept in these solvents or dichloromethane.

There was no loss of analytes from the plasma or urine extracts whether the organic solvents were evaporated under a light stream of nitrogen or in a Speed Vac concentrator. However, if residues from nitrogen-evaporated samples were kept at room temperature, more than 60% of 7α-thiomethyl-SL was lost within 3 h. Storage of nitrogen-evaporated samples at −20°C until their HPLC analysis prevented this degradation.

Although the extraction of 7α-thio-SL was highly variable (no reliable standard curve could be obtained), it was not detected in any plasma or urine samples of spironolactone-treated volunteers. Epoxyprogesterone is a more polar internal standard than those used in previous studies, and it does not interfere with any of the analytes nor with endogenous substances from blank or spironolactone-treated human plasma or urine. The sequential utilization of both C_{18} and CN bonded phases was absolutely necessary to eliminate endogenous contaminants.

The analytical recovery of spironolactone and its metabolites at low (25 ng/ml) and high (200 ng/ml) plasma concentrations was assessed (Table I). Although some decrease in the extraction efficiency occurred at the lower concentration, recoveries for all analytes were greater than 80% in plasma. The same effect was observed for urine standards containing the analytes at concentrations of 62.5 and 1000 ng/ml. Recoveries for all analytes were greater than 72%.

A regression analysis of the peak-height ratio *versus* concentration showed linearity over the 6.25–400 ng/ml range for plasma and 31.25–2000 ng/ml for urine. The correlation coefficient (r^2) was greater than 0.990 for all standard curves but one (Table II). The limit of quantification for all

TABLE II

LINEAR REGRESSION PARAMETERS OBTAINED FROM THE CALIBRATION CURVES OF SPIRONOLACTONE AND ITS METABOLITES IN PLASMA AND URINE

Compound	Slope	Intercept	r^2
Plasma[a] (6.25–400 ng/ml)			
Spironolactone (SL)	0.01334	0.00779	0.996
7α-Thiomethyl-SL	0.00817	0.05355	0.983
6β-Hydroxy-7α-thiomethyl-SL	0.01193	0.08532	0.991
Canrenone	0.03046	0.10760	0.993
Urine[b] (31.25–2000 ng/ml)			
Spironolactone (SL)	0.00076	0.00628	0.991
7α-Thiomethyl-SL	0.00071	−0.00182	0.993
6β-Hydroxy-7α-thiomethyl-SL	0.00052	−0.00133	0.990
Canrenone	0.00037	0.00817	0.998

[a] Concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml SL and its metabolites were analyzed in eight replicates.

[b] Concentrations of 31.25, 62.5, 125, 250, 500, 1000 and 2000 ng/ml SL and its metabolites were analyzed in eight replicates.

TABLE III

PRECISION AND REPRODUCIBILITY OF THE ASSAY FOR SPIRONOLACTONE AND ITS METABOLITES IN HUMAN PLASMA AND URINE

Sample	*n*	Spiked (ng/ml)	Coefficient of variation (%)			
			SL	II	III	IV
Within-assay						
Plasma	4	25	5.6	4.8	6.6	6.8
	4	200	1.6	4.0	4.0	0.8
Urine	6	62.5	9.1	7.5	8.9	6.1
	6	1000	4.5	2.1	8.2	4.5
Between-assay						
Plasma	8	25	11.1	6.5	12.5	10.0
	8	200	3.6	4.7	3.6	5.0
Urine	8	62.5	5.8	7.6	3.4	2.3
	8	1000	4.0	3.3	4.8	1.8

TABLE IV

ACCURACY OF THE ASSAY FOR SPIRONOLACTONE AND ITS METABOLITES IN HUMAN PLASMA AND URINE

Spiked concentration (ng/ml)	Estimated concentration (ng/ml)			
	SL	II	III	IV
Plasma				
10.9	12.7	13.9	12.9	11.3
15.6	17.3	16.7	17.8	16.3
18.8	14.8	18.1	14.7	20.9
31.3	33.7	29.7	33.7	34.2
43.8	39.9	42.5	47.6	44.1
50.0	56.1	42.5	50.0	56.3
100.0	93.8	87.8	96.0	101.2
200.0	177.1	166.8	174.2	172.0
250.0	255.0	239.9	241.5	237.4
500.0	496.3	488.5	475.5	461.3
Accuracy[a]	100.3	97.5	103.8	101.8
	12.0	12.7	9.8	8.6
Urine				
175.0	169.7	207.9	172.3	217.9
290.0	269.1	308.5	273.8	352.6
339.0	312.2	314.1	327.4	409.2
410.0	365.8	395.1	347.7	468.5
520.0	458.7	499.9	502.7	571.0
876.0	768.3	781.7	819.8	888.5
900.0	756.9	763.9	811.0	913.2
1500.0	1209.5	1240.4	1313.1	1409.3
1860.0	1580.8	1586.8	1534.7	1751.6
2000.0	1649.0	1644.0	1774.6	1845.8
Accuracy[a]	87.9	93.5	91.3	107.4
	5.1	11.7	5.4	12.4

[a] Mean percent of nominal value (± S.D.).

compounds was 6.25 ng/ml in plasma and 31.25 ng/ml in urine.

The intra-assay coefficients of variation were consistently less than 6.8% for plasma and 9.1% for urine samples (Table III). Slightly larger variations in plasma (less than 12.5%) than in urine (less than 7.6%) concentrations were found when assayed on eight different occasions over a two-month period.

Accuracy of the assay was confirmed by determining ten different concentrations in plasma and urine (Table IV). The accuracy was defined as the overall range of mean percentage differences from nominal and was comprised between −12.1% and 7.4% for all compounds in plasma or urine.

The sturdiness of the method was demonstrated by analyses of plasma and urine samples obtained from both clinical and animal studies. Fig. 3 shows representative chromatograms (at 254 nm) of processed plasma samples. Fig. 3A shows a drug-free plasma spiked with internal standard and 50 ng/ml spironolactone and metabolites II, III and IV. Sensitivity of canrenone was greatly enhanced at 280 nm (chromatogram not shown). Fig. 3B shows a blank plasma from a healthy subject. The chromatogram indicates that there are no apparent interferences from endogenous compounds. Fig. 3C shows a plasma sample taken 90 min after the oral administration of a 200-mg dose of spironolactone to the same subject.

Although the peak between metabolite III and SL has the same retention time as one of the degradation products of metabolite II (double arrowheads), it remains to be determined whether or not they are structurally identical. Fig. 4 shows representative chromatograms of processed urine samples from a healthy subject. Fig. 4A shows drug-free urine spiked with internal standard and 125 ng/ml spironolactone and its metabolites. Fig. 4B shows an urine sample from a healthy subject. No interfering peaks from endogenous compounds were observed. Fig. 4C shows an urine sample taken 8 h after the oral administration of a 200-mg dose of spironolactone to the same subject. Under our chromatographic conditions, all peaks of interest were well separated. Urinary levels of canrenone were elevated enough at 254 nm that quantification at 280 nm was not deemed necessary. The plasma concentration–time profile for SL and its metabolites after the administration of an oral dose of 200 mg of spironolactone given to a fasting healthy male volunteer is presented in Fig. 5. The time course of urinary concentrations of SL and its metabolites for the same volunteer is presented in Fig. 6.

CONCLUSION

An extraction procedure based on two sequential solid-phase extractions has been validated for the determination of SL and its metabolites in plasma and urine. It provides a rapid, selective and reliable method for SL and its metabolites in biological fluids. The suitability of this method was demonstrated in pharmacokinetic studies of

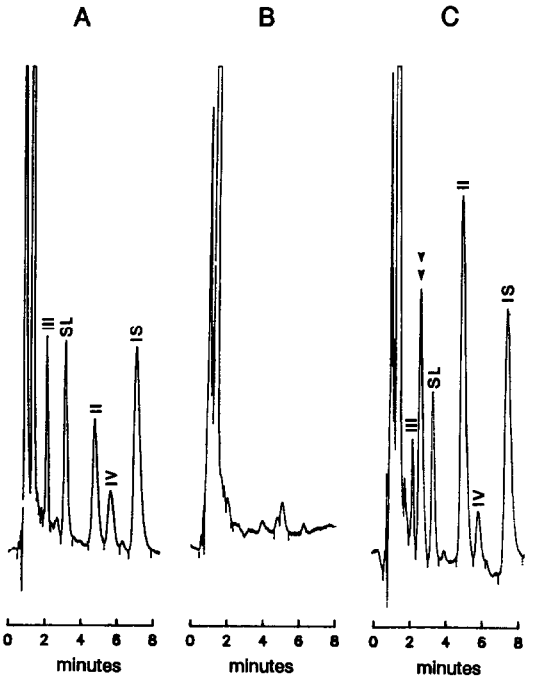

Fig. 3. Chromatograms of extracts obtained from 1.0 ml of human plasma. (A) Drug-free sample spiked with 50 ng/ml SL (3.0 min), II (4.6 min), III (2.0 min) and IV (5.4 min); (B) sample obtained immediately before and (C) sample obtained 90 min after the administration of a 200-mg oral dose of SL to a healthy volunteer. Internal standard (100 ng) was added to the plasma samples in A and C. An unknown peak (double arrowheads) with a retention time of 2.5 min appears between the metabolite III and SL.

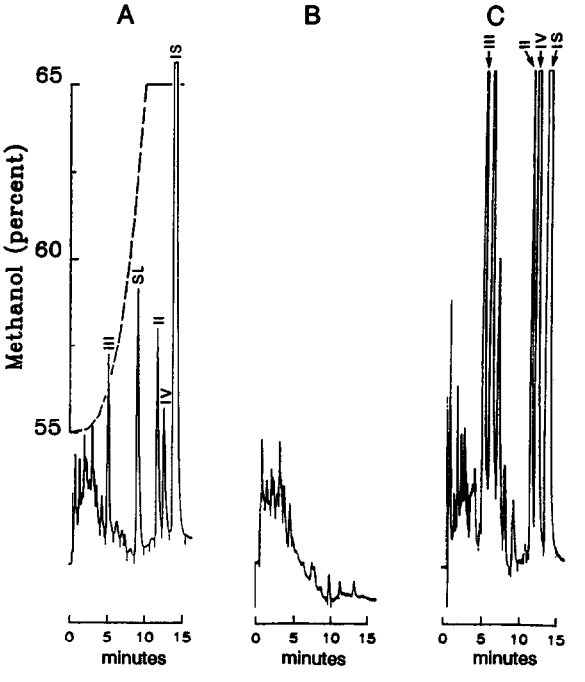

Fig. 4. Chromatograms of extracts obtained from 1.0 ml of human urine. (A) Drug-free urine spiked with 125 ng/ml SL (8.3 min), II (10.9 min), III (4.5 min) and IV (11.7 min); (B) sample obtained immediately before and (C) sample obtained 8 h after the administration of a 200-mg oral dose of SL to the same volunteer as in Fig. 3. Internal standard (1000 ng) was added to the urine samples in A and C. Unknown peaks with retention times of 5.5 and 6.5 min appear after the metabolite III. The time course of methanol concentration in the mobile phase is shown in A (dashed line).

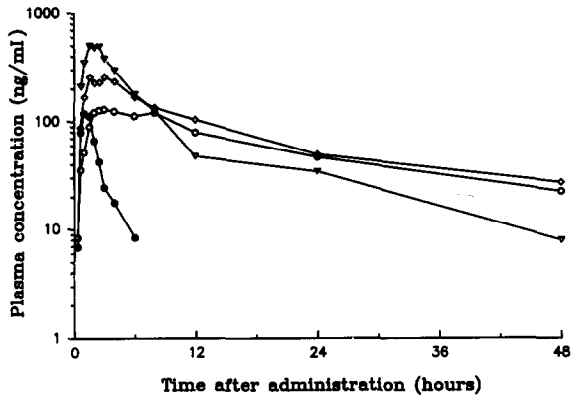

Fig. 5. Time course of plasma concentrations for SL (●), 6β-hydroxy-7α-thiomethyl-SL (○), 7α-thiomethyl-SL (▽) and canrenone (◇) after an oral dose of 200 mg SL to a healthy volunteer.

SL and its metabolites in humans. Moreover, this method may prove useful for further investigation on the biotransformation of SL since it allows the chromatographic separation of yet unidentified degradation products that could be metabolites of SL.

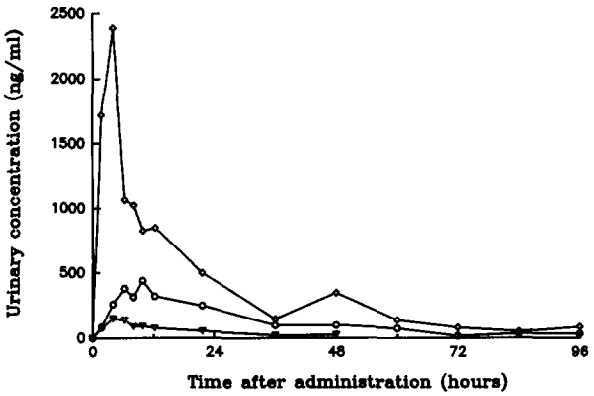

Fig. 6. Urinary concentrations of 6β-hydroxy-7α-thiomethyl-SL (○), 7α-thiomethyl-SL (▽) and canrenone (◇) after an oral dose of 200 mg SL to the same volunteer as in Fig. 5.

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