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DETERMINATION OF AN ALDOSTERONE ANTAGONIST IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

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SUMMARY

An automated high-performance liquid chromatographic assay for the determination of an aldosterone antagonist (I) is described using column switching for direct injection of urine samples. After dilution with buffered internal standard solution, the sample was injected onto a clean-up column (17 × 4.6 mm I.D.), dry-packed with C_{18} reversed-phase material (particle size 30 μm). Polar urine components were removed by flushing the clean-up column with water. Retained substances, including I and the internal standard, were desorbed by backflush elution onto a 5-μm ODS-silica analytical column (125 × 4 mm I.D.), separated with water—methanol—tetrahydrofuran, and detected at 295 nm. After backflushing the analytical column and re-equilibrating the clean-up column, the system was ready for the next injection. The limit of quantification was ca. 100 ng/ml, using a 100-μl specimen of diluted urine. The mean inter-assay precision of the method up to 25.6 μg/ml was 2%. Practicability and accuracy of the new method were demonstrated by the application to excretion studies performed with human volunteers.

INTRODUCTION

5-Oxo-A-tetranor-17α-pregna-9,11-diene-21,17-carbolactone (Ro 14-9012, F. Hoffmann-La Roche, Basle, Switzerland) (I, Fig. 1) is currently under clinical investigation as a new aldosterone antagonist. For pharmacokinetic studies performed in man, a precise method for the determination of urine levels of

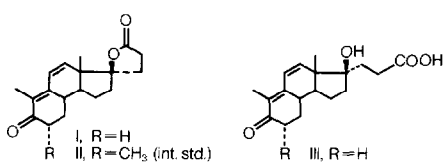

Fig. 1. Chemical structures for the compounds referred to in the text.

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the lactone I in the presence of metabolite III was required. Preliminary experiments showed that the open form III may convert back to the parent compound during "conventional" sample preparation steps, leading to falsely high measurements of I. Therefore, a fully automated column-switching method was developed avoiding any critical sample pretreatment steps. The new assay has been successfully applied to more than 100 urine samples from a tolerance study in man.

EXPERIMENTAL

Reagents and solvents

Methanol (HPLC grade), sodium dihydrogen phosphate (p.a.) and disodium hydrogen phosphate (p.a.) were obtained from Fluka (Buchs, Switzerland). Tetrahydrofuran (HPLC grade, unstabilized) was purchased from Fisons (Loughborough, U.K.). Double-distilled water was used as mobile phase 1 and for preparation of mobile phase 2. The internal standard solution was prepared by spiking a sodium phosphate buffer solution (0.5 *M*, pH 7.5) with II at a concentration of 2 μg/ml.

Calibration samples

A stock solution a of 2.56 mg of I in 1 ml of methanol was prepared by ultrasonication. Working solutions b—e, containing 640, 160, 40 and 10 μg/ml, were obtained by diluting aliquots of the stock solution with methanol. The calibration samples A—E, containing 25.6, 6.4, 1.6, 0.4 and 0.1 μg/ml, were prepared by diluting 0.2 ml of the corresponding solutions a—e with human blank urine to 20 ml. The stock solution was stable for at least three months at 0—4°C. Working solutions were prepared each time prior to use. The calibration samples were divided into aliquots of 1 ml and stored deep-frozen (—20°C) until required for analysis.

Quality-control samples

Quality-control (QC) samples containing 6.4, 1.6, 0.4 and 0.1 μg/ml were prepared by diluting 0.2 ml of methanolic working solutions (640, 160, 40 and 10 μg/ml) with pretested human blank urine to 20 ml. The QC samples were divided into aliquots and stored deep-frozen (—20°C) until required for analysis.

To obtain an optimum control of the assay, calibration samples and QC samples were prepared by different persons using different stock solutions.

Instrumentation

A schematic representation of the column-switching system is given in Fig. 2. A single-piston pump (Model 110A; Altex-Scientific, Berkeley, CA, U.S.A.) was used as pump P1 delivering double-distilled water as purge solvent at a flow-rate of 2 ml/min. Aliquots (20—100 μl) of diluted urine samples were injected by an automatic sample injector (I1; Model WISP 710A; Waters, Milford, MA, U.S.A.) onto the clean-up column (C1; 17 × 4.6 mm I.D.; Bischoff-Analysentechnik, Leonberg, F.R.G.), dry-packed with Nucleosil 30 C_{18} (Macherey-Nagel, Düren, F.R.G.). A holder (F) for replaceable frits of

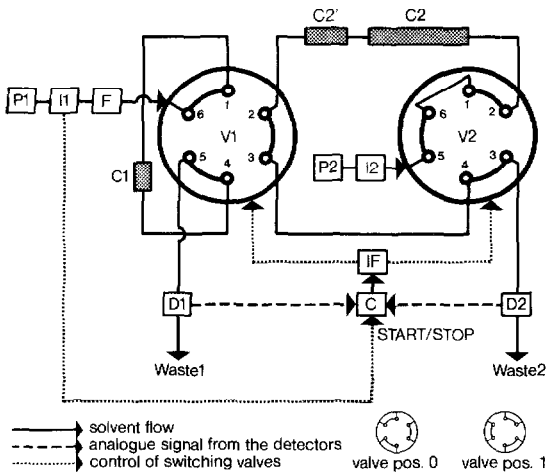

Fig. 2. Schematic representation of the column-switching system assembled for the analysis of I in urine (see text for details).

0.5 μm pore diameter (Type 7335; Rheodyne, Cotati, CA, U.S.A.) was placed between the automatic injector and the first switching valve (V1). A detector (D1; Pye Unicam, Cambridge, U.K.) operating at 254 nm was used to monitor the removal of urine components from the clean-up column during the purge step. The pump (P2; Model 110A; Altex) delivered the mobile phase at a flow-rate of 1 ml/min for backflush elution of the retained compounds from the clean-up column onto the analytical column (C2; 125 × 4 mm I.D.; Merck, Darmstadt, F.R.G.), slurry-packed with 5-μm Hypersil ODS (Shandon, Astmoor, U.K.). A guard column (C2'; 30 × 4 mm I.D.; Merck) filled with the same material was used to protect the analytical column. The composition of the mobile phase was water—methanol—tetrahydrofuran (200:175:25). A manual injector (I2; Model 7125; Rheodyne) was used for injection of the control solutions directly onto the analytical column. Detection of the eluted compounds was carried out at 295 nm with a variable-wavelength detector (D2; Pye Unicam).

The two air-actuated switching valves (V1, V2; Model 7000A; Rheodyne), assembled with two solenoid valves (Model 7163; Rheodyne), were controlled by the external time events of a computing integrator (C; Model SP 4200; Spectra Physics, San José, CA, U.S.A.). However, the 30 mA/TTL compatible output of the integrator was not suitable for directly activating the solenoid valves, which had an input characteristic of 400 mA per 12 V DC. Therefore, to achieve compatibility, a cheap home-made interface (IF) was placed between the integrator output and the solenoid valve input.

Acquisition, reduction and documentation of the data were performed by means of the computing integrator (C) working with a modified version of a BASIC program recently developed for the Model SP 4100 computing integrator [1].

Analytical procedure

The samples required only a minimum of pretreatment: an aliquot of 0.2

ml of urine sample was mixed with 0.2 ml of buffer solution containing the internal standard. After centrifugation (15 min at 1700 *g*), the sample was ready for chromatography.

A complete automated sample analysis required a total of 28 min and included the following four column-switching steps.

Step A (V1 = 0, V2 = 1; 7 min). An aliquot (20—100 μl) of buffered urine sample was injected onto the clean-up column (C1). Polar urine components were washed with water via D1 to waste 1, while less polar compounds, including I and II, were retained on the clean-up column. The analytical column (C2) remained in the backflush mode in order to remove strongly retained substances of the previous run.

Step B (V1 = 0, V2 = 0; 3 min). The analytical column was prepared for the subsequent step by reversing the flow of mobile phase 2 to the normal direction. The purge process continued on the clean-up column.

Step C (V1 = 1, V2 = 0; 12 min). Enriched material was transferred from the clean-up column onto the analytical column in the backflush mode by means of the mobile phase delivered by pump P2. After chromatographic separation, I was quantified by means of detector D2.

Step D (V1 = 0, V2 = 1; 6 min). The clean-up column was switched back into the stream of pump P1 in order to re-equilibrate the reversed-phase material for the next sample injection. At the same time, strongly retained compounds were removed from the system by backflushing the analytical column.

Calibration and calculation

Together with the biological samples, five calibration samples covering the expected concentration range were processed as described before. The calibration curve was obtained by weighted linear least-squares regression (weighting factor = $1/y^2$) of the measured peak-height ratios I/II (y) versus the concentrations of I (x) added to the urine. This curve was then used to interpolate concentrations of I in QC samples and unknowns from the measured peak-height ratios I/II.

Preliminary extraction procedure

Initially, a conventional extraction method for the determination of I in urine was developed, but this was given up later for reasons pointed out in the discussion part of this report.

Urine samples were diluted with water (1:5), mixed with II as internal standard and extracted twice with *n*-butyl chloride—dichloromethane (96:4). After centrifugation, the organic layers were separated, combined and evaporated to dryness at 50°C by means of a gentle stream of nitrogen. The reconstituted extract was chromatographed on a reversed-phase system (Hypersil ODS, 5 μm; water—methanol—tetrahydrofuran, 180:190:30) with UV detection of the effluent at 295 nm.

RESULTS

Precision

The precision of the method was evaluated over a concentration range of

TABLE I

PRECISION OF THE COLUMN-SWITCHING PROCEDURE

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Difference between found and added concentration (%)
Intra-assay precision (n=5)			
50*	51.3 ± 1.07	2.1	+2.6
100	105 ± 6.25	6.0	+5.0
400	423 ± 2.47	0.6	+5.8
1600	1630 ± 21.2	1.3	+1.9
6400	6330 ± 48.1	0.8	−1.1
25600	25400 ± 333	1.3	−0.8
Inter-assay precision (n=7)			
50*	46.6 ± 4.71	10.1	−6.8
100	99.1 ± 4.73	4.8	−0.9
400	416 ± 6.69	1.6	+4.0
1600	1630 ± 23.7	1.5	+1.9
6400	6300 ± 31.7	0.5	−1.6
25600	25000 ± 388	1.6	−2.3

*Below limit of quantification (100 ng/ml).

0.05—25.6 μg/ml. The intra-assay precision was determined by analysing each concentration five times on the same day. The inter-assay precision was obtained by analysing the samples on seven days over a period of two weeks (using a separate calibration for each day). The data shown in Table I demonstrate the high degree of precision of the fully automated high-performance liquid chromatographic (HPLC) method.

Limit of quantification

The detection limit, defined by a signal-to-noise level of ca. 3:1, was < 50 ng/ml, using a 100-μl specimen of diluted urine. However, several months experience with the new method showed that a practical limit of quantification of 100 ng/ml was more realistic with respect to acceptable accuracy and precision at low concentrations (Table I).

Selectivity

Several blank urine samples from different human volunteers were analysed as described. No interferences from endogenous components present in the urine could be observed. Fig. 3a presents a typical chromatogram of human urine collected before application.

Linearity

A linear correlation between peak-height ratio I/II versus concentration of I was found up to 25.6 μg/ml. According to Table I, the standard deviations and, therefore, also the variances were not constant over this wide concentration range. For this reason, the calibration curve had to be calculated by means of a weighted linear least-squares regression procedure, using $1/y^2$ as weighting

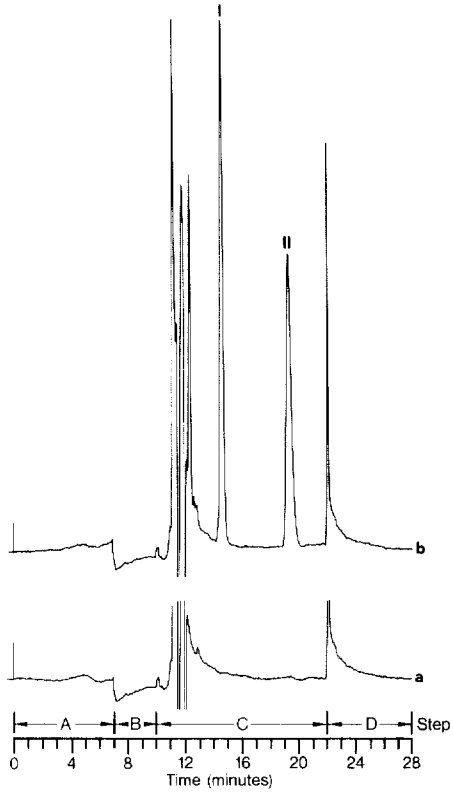

Fig. 3. Chromatograms of human urine samples: (a) before application; (b) collected 0–2 h after a single oral dose of 600 mg of I; measured concentration, 2.23 μg/ml. Detector D2, λ = 295 nm, range 0.02; recorder sensitivity, 20 mV; injection volume, 100 μl.

TABLE II

STABILITY OF I IN HUMAN URINE (n = 6)

Sample	Concentration (ng/ml)	Change of concentration after storage (%)	90% Confidence interval (%)
Control*	200	—	—
24 h/25°C	201	+0.5	−4.3 to +5.5
Three months/−20°C	194	−2.9	−5.7 to 0.0
Control	2000	—	—
24 h/25°C	1970	−1.7	−4.4 to +1.1
Three months/−20°C	1960	−2.0	−3.6 to −0.3
Control	20000	—	—
24 h/25°C	20100	+0.6	−1.7 to +3.0
Three months/−20°C	19400	−2.8	−5.1 to −0.5

*Freshly prepared.

factor [2]. The standard software of the computing integrator provided only conventional linear regression and had, therefore, to be modified by means of additional programs [1].

Stability of I in urine

Control urine was prepared at concentrations of 0.2, 2 and 20 μg/ml. One portion of these samples was stored at room temperature for 24 h and then analysed. The other portion was frozen, stored at −20°C for three months and then analysed. With each set of stored samples, an equal number of freshly prepared samples was analysed to provide the 100% values. The statistical interpretation of the data followed the procedure recently developed [3]. The data presented in Table II indicate that I was stable in urine under the storage conditions investigated.

DISCUSSION

Preliminary extraction method

Initially, a preliminary HPLC method involving conventional extraction was developed, which gave satisfactory results for spiked urine samples. Surprisingly, poor results were obtained when the method was applied to samples from a human tolerance study. Coefficients of variation of up to 38% were obtained for triplicate determinations, while analyses of QC samples showed precise results (Table III), indicating that the synthetic QC samples did not properly reflect the conditions present in the real samples. It was assumed that the biological samples contain an unstable metabolite, which is partly converted back to the parent compound during storage and/or handling of the samples. Metabolic investigations [4] showed that the acid III was present in urine samples of dogs treated with I and may, therefore, also occur in human urine. Because I was found to be stable in urine (see Table II), we assumed that III was formed by the metabolism of I, rather than during sample storage. In-

TABLE III

URINE CONCENTRATIONS OF I FOR SUBJECT M.H. FOLLOWING A SINGLE ORAL DOSE OF 100 mg OF I MEASURED BY TWO DIFFERENT METHODS

Sample	Extraction method		Column-switching method	
	Mean concentration (μg/ml)	Coefficient of variation (%)	Mean concentration (μg/ml)	Coefficient of variation (%)
M.H./ 0— 2 h*	1.79	8.1	0.79	2.7
M.H./ 2— 5 h	6.49	10.9	2.39	0.9
M.H./ 5— 8 h	3.26	21.4	0.81	1.7
M.H./ 8—12 h	1.29	38.3	0.40	5.4
M.H./12—24 h	0.93	16.0	0.67	3.2
QC/6.4 μg/ml**	6.59	2.6	6.37	4.1
QC/1.6 μg/ml	1.64	1.5	1.61	3.5

*Urine sample of subject M.H., collected 0—2 h after administration.
**Quality-control sample containing 6.4 μg/ml I in blank urine.

vestigations with the extraction method showed that III was partly co-extracted from unbuffered urine and then, to some extent, converted to I during the following steps, leading to falsely high measurements of the lactone.

The objective of the following investigations was, therefore, to develop a procedure which allowed the precise measurement of the lactone I in the presence of its open form III. As an alternative to improving the preliminary extraction procedure, a new column-switching method was developed, which avoided any critical sample preparation steps such as extraction, evaporation, reconstitution, etc. Re-analyses of the samples then resulted in much lower concentrations of I than previously obtained with the extraction method, as demonstrated by the results in Table III. Additionally, the precision was better than obtained with the preliminary extraction method, and similar to that obtained for the QC samples.

Pretreatment of samples

The open form III was not stable under acidic conditions, while conversion into the lactone was negligible at a slightly basic pH (Table IV). Therefore, to suppress conversion of III to I during storage of the samples in the automatic injector, the urine had to be stabilized by dilution (1:1) with 0.5 *M* phosphate buffer (pH 7.5) containing II as internal standard. Furthermore, the samples were centrifuged before injection in order to prevent blockage of the filter assembled between injector 1 and switching valve 1.

TABLE IV

STABILITY OF III IN PHOSPHATE BUFFER AT ROOM TEMPERATURE

Incubation time (h)	Formation of I (%) after incubation of III (10 μg/ml) in 1/15 *M* buffer solutions				
	pH 4	pH 5	pH 6	pH 7	pH 8
2	4.6	1.2	0.6	0.6	0.6
5	10.2	1.9	1.0	0.8	0.6
24	37.3	7.1	2.5	1.5	1.2

Column-switching process

The optimal purge time for complete removal of polar, ultraviolet-absorbing urine components from the clean-up column was determined by injecting urine samples onto the system and monitoring the purge process by means of detector D1. Using a purge flow-rate of 2 ml/min, the clean-up process was completed in < 10 min (Fig. 4).

The recovery of I from buffered urine was determined by replicate analysis of a spiked urine sample by means of column switching, followed by injection of a corresponding aqueous solution directly onto the analytical column, the latter analysis providing the 100% value. A recovery of $> 90\%$ was found, indicating that enrichment on the clean-up column, isolation from matrix constituents, and transfer onto the analytical column occurred almost without loss of substance and with negligible peak broadening.

The efficiency of automated HPLC procedures is much improved by the removal of late-running compounds from the analytical column. To this end, gradient elution is often applied, choosing a final solvent mixture of high

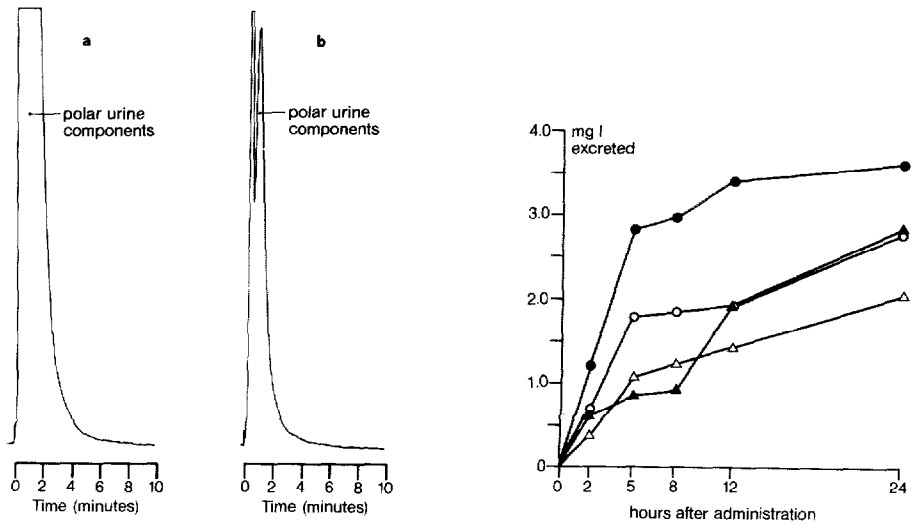

Fig. 4. Control of the purge process after injection of (a) 100 μl and (b) 20 μl of human urine by means of the monitor detector D1. Detector D1, λ = 254 nm, range 0.02; recorder sensitivity, 50 mV.

Fig. 5. Cumulative renal excretion of unchanged compound after oral administration of 100 mg of I to four healthy volunteers.

solvent strength [5, 6]. In the system described here, however, the analytical column was cleaned by simply reversing the flow of mobile phase 2 for 13 min in order to backflush strongly retained compounds to waste 2. One stated disadvantage of this procedure is the stress applied to a column when the flow is continually reversed, resulting in a decrease in column lifetime. However, for the procedure described here, it was possible to analyse at least 400 urine samples without any deterioration being observed.

It was advisable to protect the analytical column by an additional guard column, which was exchanged after 100—200 injections. The clean-up column could be used for up to 400 injections. From time to time, the filter frit in the holder also had to be exchanged in order to avoid a high back-pressure at pump P1.

External standard, internal standard

Bioanalytical methods, involving a conventional sample preparation procedure, normally make use of an internal standard in order to minimize inaccuracies caused by pretreatment, injection, chromatography and quantification of the samples. HPLC column-switching methods avoid classical pretreatment steps, make use of precise loop injectors and, therefore, often do not need an internal standard to improve precision and accuracy of the measurements [7, 8].

However, external standard methods are sensitive to parameters which might change slightly during chromatography and detection, such as performance of the columns, temperature and composition of the mobile phase, sensitivity of the detector, etc. In particular, a decrease of overall performance of the combination of columns with time is a critical point in column-switching systems. The packing of the clean-up column is permanently stressed by

repetitive changes of pressure, flow direction and composition of the mobile phase, and there is also a tendency for some endogenous components present in the biological fluids to be irreversibly bound to the clean-up column [9].

Therefore, to compensate slight peak-height changes during the run, the methyl analogue of I was used as internal standard. Furthermore, the stability of the HPLC system was monitored by QC samples which were analysed at the beginning and end of a sample sequence. Several months' experience with the new method showed that, compared with external standardization, the quality of the measurements was generally improved by use of the internal standard, especially when long sample sequences were processed.

Application of the method to biological samples

The new column-switching method has been successfully applied to the analysis of > 100 urine samples from a tolerance study in man. Fig. 3 shows two representative chromatograms from this study.

Compound I was orally administered in different doses to healthy male volunteers. Plasma samples were collected at different times and total urine collections were made during specific periods. The concentrations of I in the urine were measured in duplicate on two different days. The mean coefficient of variation was 1.9% and was not concentration-dependent. The amount of I excreted in the urine was obtained by multiplying the concentration by the volume of the collected fraction.

The renal excretion was small: < 5% of the dose after 100 mg of I (Fig. 5). The amount of I excreted in the urine increased proportionally to the dose after 100 and 300 mg, but not after 600 mg [10].

The renal clearance of I was the same after 100 and 300 mg (median: 16.8 and 17.2 ml/min), but smaller after 600 mg (median: 11.3 ml/min). The difference was significant (Wilcoxon-Test: $2\alpha = 0.05$).

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