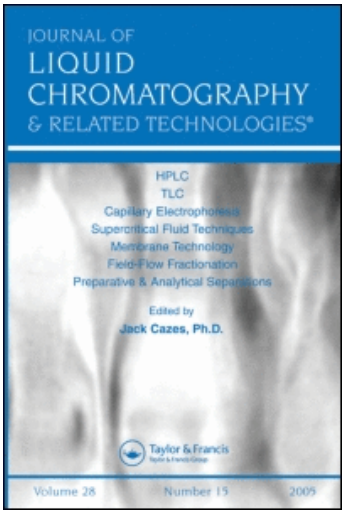

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M. Bidart[a]; G. Lesgards[b]
[a] DCN - Toulon CTSN/MDTC/HSE Laboratoire de Biotoxicologie, Toulon Naval, BP, France [b] Faculté des Sciences et Techniques de St. Jérome Avenue Escadrille, Marseille, Cedex, France

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DIRECT INJECTION ANALYSIS OF 6β-HYDROXYCORTISOL AND CORTISOL IN URINE BY HPLC-UV WITH ON-LINE ISRP PRECOLUMN

M. BIDART[1] AND G. LESGARDS[2]
[1]DCN - Toulon
CTSN/MDTC/HSE
Laboratoire de Biotoxicologie
BP 28, 83800 Toulon Naval, France
[2]Faculté des Sciences et Techniques de St. Jérome
Avenue Escadrille Normandie-Niemen
13397 Marseille Cedex 13, France

URINARY STEROIDS DIRECT INJECTION

The simultaneous measurement of 6β-hydroxycortisol (6β-OHF) and cortisol (FF) is interesting for the evaluation of enzyme induction in man. An on-line HPLC-UV analysis of urinary steroids is described. In a first step, the biological sample was injected onto an ISRP (Internal Surface Reversed Phase) precolumn with water for the elimination of proteins and indesirable products and for the concentration of hydrophobic molecules. In a second step, a simple gradient of acetonitrile (ACN) in water, by a backflush procedure, eluted the retained analytes which are analysed by conventional RP-HPLC coupled with UV detection.

INTRODUCTION

The importance of measuring 6β-hydroxycortisol as an index for studying microsomal enzyme induction of cytochrome P-450 3A (CYP 3A) is well recognised to explore the action of some drugs or foreign chemicals compounds (1-3).

Many HPLC procedures for the determination of steroids in biological fluids have been described last years. With regard to 6β-OHF analysis some techniques have been developped using either normal phase (3-8) or reversed phase (9-14). Very often, these methods need an important treatment of the sample, with liquid-liquid extraction (3, 5, 9, 12) or adsorption on silica cartridges (4, 8, 11), joined alkalin and, sometimes, acid washings.

We have developped an alternative liquid chromatography which suppress the step of extraction and the employment of internal standard. This technique use a backflush system with a ten-port valve and ISRP precolumn, concept designed for the time by Hagestam and Pinkerton (15) and reported for some applications as quantification of drugs (16-18). The recovery of analytes is 100%.

MATERIAL AND METHODS

Steroid Standards

Cortisol, cortisone, corticosterone, 11-deoxycortisol, desoxycorticosterone, prednisone and prednisolone were obtained from Sigma Chemicals. 6β-hydroxycortisol was purchased from Steraloids (distributed in France by Touzard et Matignon).

Methanolic stock solutions of each steroid at 1.000 g/l were stored at 4°C. Daily, fresh dilutions were prepared in mobile phase.

Solvents

Methanol and acetonitrile were purchased from Carlo Erba (HPLC quality).

Chromatography

Initial conditions were leaded by the method developped in our laboratory for the analysis of cortisol and cortisone in saliva (19).

The chromatographic equipment is a fully automatised system purchased from Spectra Physics (now Thermo Instrument Products) with :

- quaternary pump model P-4000, with solvent degazer (porous membranes)
- sample preparator autoinjector AS-3000, thermostat equipped
- detector focus 2000 scanning from 190 to 800 nm
- interface SN 4000.

Data were collected and evaluated with a Spectra Physics PC 1000 software on a Getek 486 computer.

Mobile phase was delivered at a ten-port valve (select-sil 99T) with manual or electropneumatic command (figure 1).

In position 1, the sample was flushing with water into a ISRP cartridge (Ultrabiosep C_{18}, particules of 10 µ, SFCC), in an optimal time of 5 minutes. In position 2, with a backflush system, all the retained molecules were dissolved in mobile phase (ACN / water), separated into an analytical column Ultrabase C_{18} (250 X 4.6 mm, 5 µ, SFCC) and analysed with a scan mode from 235 to 254 nm.

With isocratic mode, some assays were done, concentration of ACN varying between 20 and 40 %, in order to verify the comportment of all the steroids (figure 2).

Because of interferences in urine injection in the first part of chromatograms, we have choosed to work with gradient mode. To retain more 6β-OHF we have begun the chromatographic conditions

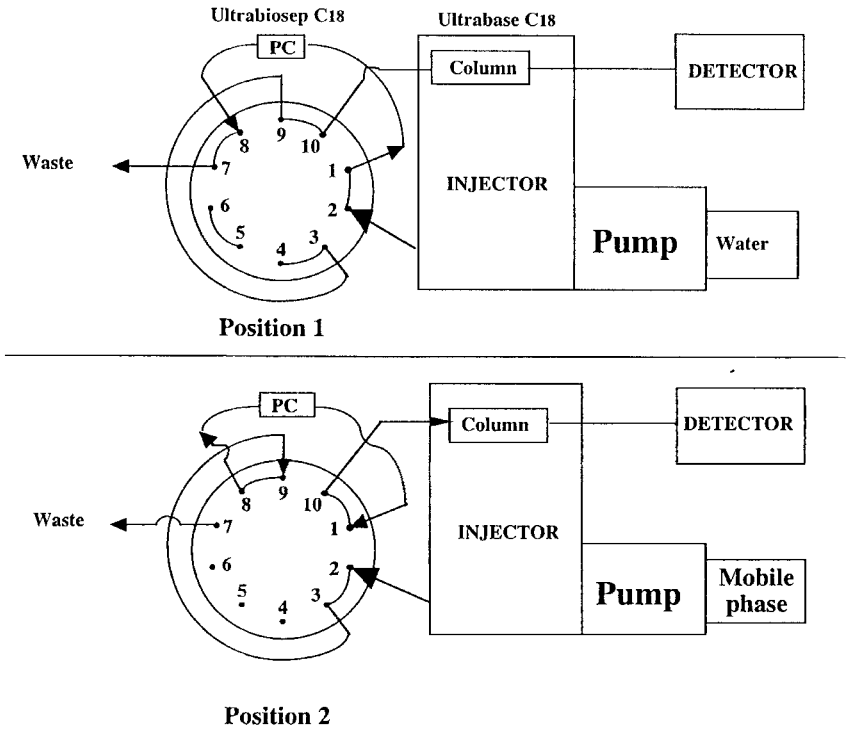

FIGURE 1 :
valve switching system for on-line urine injection.

by 20 % of ACN in water. After 6β-OHF elution, this percentage was rapidly increased to 40 for the quick elution of the other steroids. Some different assays concerning the changement of concentration indicated the optimal time at 15 min. After 25 min., we reconditioned the column to the initial percentage of 20 for 5 min. At least, we came back to position 1 of the valve to sweep precolumn in water for 5 min.

The scheme of final chromatographic conditions are given in figure 3 and table 1.

The system was ready for a new injection.

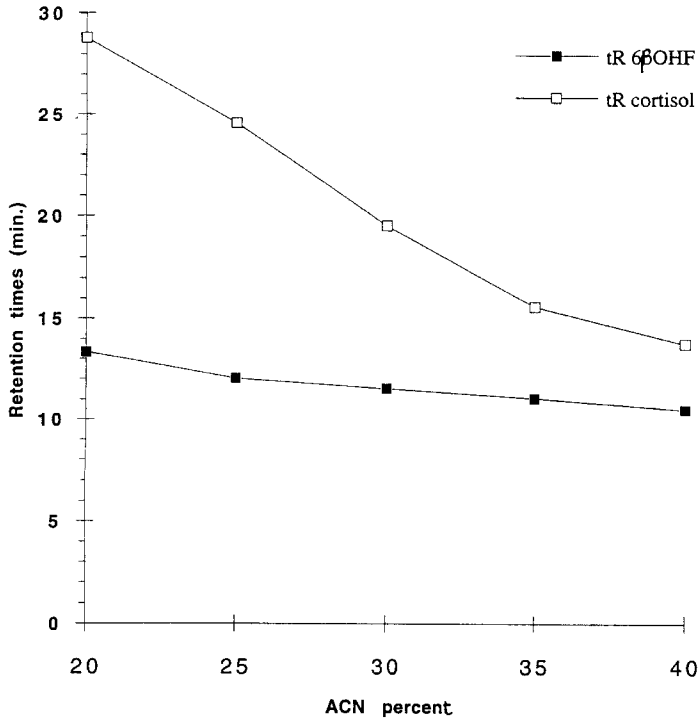

FIGURE 2 :
progression of retention times of 6β-hydroxycortisol and cortisol, according to volumic concentrations of solvent.

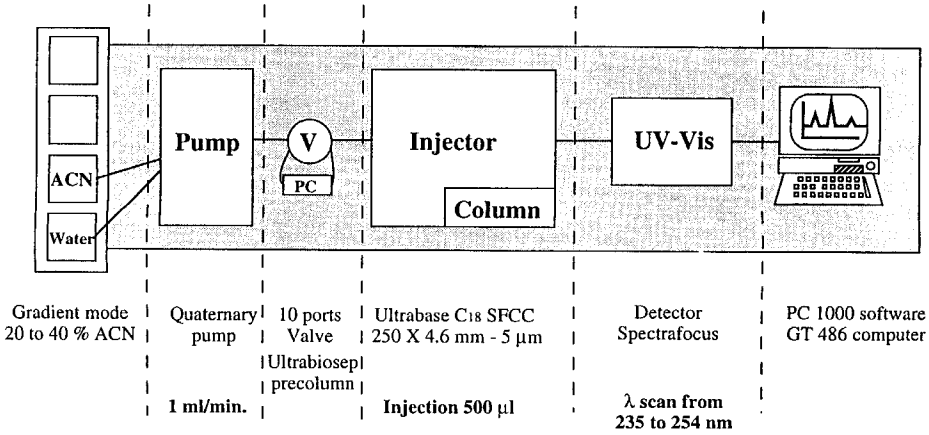

FIGURE 3 :
Chromatographic conditions for an on-line analysis of urinary steroids (LC/UV).

TABLE 1 :
Resume of the Valve Position during the chromatogram Time.

Time	ACN percent (1 ml/min.)	Valve position
0	0	1
5	0	1
5.01	20	2
15	20	2
15.01	40	2
25	40	2
25.01	20	2
30	20	2
30.01	0	1

The urine samples collected from 78 subjets were frozen and, after thawing, centrifuged at 3000 rpm and filtred into 0.22 μ membranes (Millex GS). A pool of urines served as control, in addition to standard solutions (100 and 200 μg/l), systematically injected after four biological samples.

RESULTS

The control of a standard solution with the seven steroids gave the following retention times (figure 4):

6β-OHF :	13.26 ± 0.04	(k' = 2.63)
Prednisolone :	22.58 ± 0.03	(k' = 11.94)
Cortisol :	22.77 ± 0.03	(k' = 12.13)
Cortisone :	23.11 ± 0.04	(k' = 12.47)
Prednisolone :	25.38 ± 0.04	(k' = 14.74)
Corticosterone	25.65 ± 0.05	(k' = 15.01)
11-deoxycortisol :	26.58 ± 0.05	(k' = 15.94)
Deoxycorticosterone :	27.46 ± 0.05	(k' = 16.82)

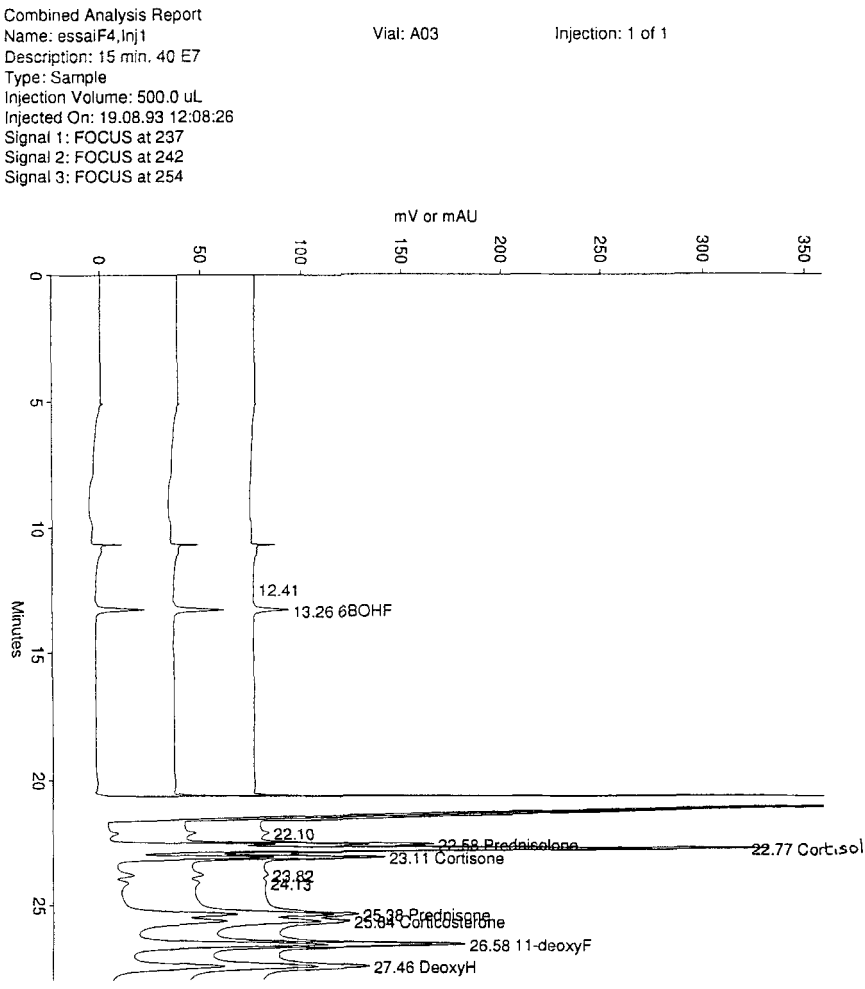

FIGURE 4 :
Chromatogram of a standard solution containing the seven steroids, with detection at 237, 242 and 254 nm.

Recovery

This technique with on-line direct injection has permitted the absolute recovery of steroid compounds.

Linearity and Precision

The response of the detector is linear for concentrations of metabolites from 0 to 5.000 mg/l. All the correlation coefficients of standard curves were equal to 0.999.

The coefficients of variation (CV) intra-assay for 6β-OHF and cortisol were included between 0.81 and 3.30 %, the best values founded at 242 nm and the worse ones at 254 nm. The CV inter-assays were always lower than 4 % for standard solutions and than 6 % for the reference urine thawed every day and treated with the other biological samples.

Detection Limit

The detection limits for 6β-OHF and cortisol were respectively 4.00 µg/l and 3.00 µg/l, that is to say for an injection of 500 µl 2.00 ng and 1.50 ng injected. The quantification limit was 10.00 µg/l for both steroids.

DISCUSSION

With this technique, we have quantified 76 urines out of 78, without any calculation (with only double injection). For the two other samplings, which were very loaded and one of them jaundiced, an interference peak at 13.56 min. obstructed the 6β-OHF one, and did't permit an accurate quantification (figure 5). We thank it was

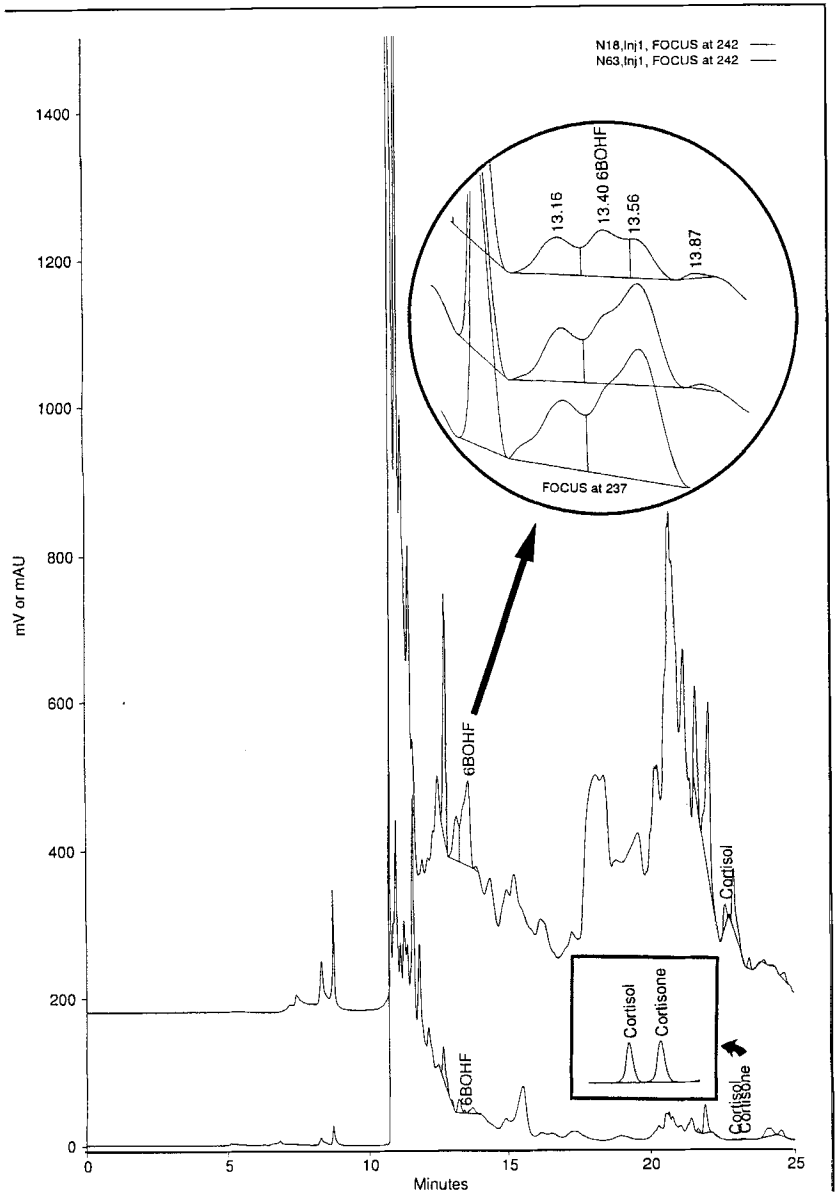

FIGURE 5 :
Chromatograms of urine samples, with direct injection on the column switching technique (LC/UV)

(a) normal urine

(b) loaded jaundiced urine with difficulties to quantity 6β-OHF, because of interference peak at 13.56 minutes.

better to extract selectively the steroid molecule than to give an approximate value with the sophistical software. For cortisol and cortisone determination, no problem occurred, all the integrations were accurated.

This technique has been evaluated by a similar method with liquid-liquid extraction procedure and the same chromatographic system.

One part of the filtred urine was extracted with 5 parts of ethylacetate, then, after centrifugation (10 min. ca 1330 g), the organic phase was washed with one part of NaOH solution 0.1N and one part of water. After removing aqueous phase, the eluate was reduced to dryness under a stream of nitrogen and the extract was reconstituted in 200 µl of mobile phase. The recoveries for 6β-OHF and cortisol were respectively 95.6 % and 92.3 % (n = 10). Chromatographic conditions were the same as described method, except isocratic mode with 30 % of ACN and the absence of the valve with precolumn.

We determined the linear regression between the two methods for 6β-OHF and cortisol with 76 or 78 urinary samples (table 2). The good correlation coefficients (r) and the test of Student applied on the slope (t) showed the quality of the regression.

The two samples with interference peak were easy to detect on the chromatograms. Also, we suggest than, with our simple technique without long and expansive treatment of the biological complex mixtures like urine or plasma, we can quantify some of important steroids and establish directly the ratio of 6β-OHF and cortisol for enzyme induction studies, with the same injection for the almost totality of the samples. If a problem occurs for the quantification of 6β-OHF, we advise the treatment of the biological fluid and the control with the extract product, relieved of interference peak. For studies about cortisol and cortisone, in urine or in saliva, the same simplified technique with isocratic mode (30 % ACN) can be used with success.

The steroids levels for the 76 urines tested (healthy male adults) were :

TABLE 2
Linear regression between HPLC-UV with on-line urine direct injection and HPLC-UV with liquid-liquid extraction of steroids by ethyl acetate

Steroid	Linear regression	n	Correlation (r)	Student (probability)
6β-OHF	Y = 1.006 X	78	0.9223	42.86 (0.000)
	Y = 0.992 X	76	0.9805	83.90 (0.000)
Cortisol	Y = 1.009 X	78	0.9945	155.15 (0.000)

mean and SD of 6β-OHF : 165 ± 30 µg / 24 H
FF : 47 ± 29 µg / 24 H
with a ratio of 5.4.

Our results are in agree with other authors who used similar techniques (20) or immunoenzymology (21).

CONCLUSION

With an automatised HPLC system and Scan detector, we can inject twice 20 samples a day. After short-time sample preparation, the technician is free for an other work. In addition, there is no manipulation of toxic solvents as ethylacetate, methylene chloride or ether. In this study, we have showed that is possible to work with ISRP concept for solvent volumic concentration about 40 %, adverse to others papers which limit this percentage to 20 (22, 23).

In conclusion, this method is simple, sensitive and reliable, and permits the simultaneous determination of 6β-OHF and cortisol for toxicological application.

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