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Journal of Chromatography B, 830 (2006) 278–285
JOURNAL OF CHROMATOGRAPHY B
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Direct determination of the ratio of tetrahydrocortisol + allo-tetrahydrocortisol to tetrahydrocortisone in urine by LC–MS–MS

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Received 25 January 2005; accepted 3 November 2005
Available online 23 November 2005

Abstract

The 11β-hydroxysteroid dehydrogenase (11β-HSD) is responsible for the interconversion of both the hormonally inactive cortisone and the active cortisol. This enzyme activity, which has implications in the pathogenesis of numerous diseases, is reflected in the ratio of tetrahydrometabolites of cortisol (allo-tetrahydrocortisol and tetrahydrocortisol) to those of cortisone (tetrahydrocortisone). Several methods have been proposed in the literature to determine such a ratio in urine. Most of them require tedious and extensive extraction and derivatization steps and make use of gas-chromatographic techniques, including gas chromatography coupled to mass spectrometry (GC–MS). We present here an alternative approach for the direct determination of such a ratio in urine by using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS–MS), based on a minimal sample treatment. Actually, the limit of detections (LODs) for pure standards in water permitted a simple dilution of the urine samples prior to the analysis, hence, an accurate optimization of the high performance liquid chromatography (HPLC) separation was needed in order to get rid of the severe influence of the urine matrix on the ionization efficiency. Besides, the nature of some interfering species was deeply investigated, as well as the suitability of some commercial deuterated steroids as internal standards. All these led to the final method, which was based on a HPLC separation on a C8 column and a ternary gradient water/methanol/acetonitrile. In parallel, an appropriate sample preparation was set up, which consisted of an enzymatic hydrolysis of the conjugated species and a followed 1:20 dilution. Preliminary measurements on real urine samples were performed as well.

Keywords: Steroids; Metabolism; Mass spectrometry; Electrospray ionization

1. Introduction

Glucocorticoids (GLCC) play an essential role in normal physiology by modulating metabolic and immune responses. Besides, their anomalous secretion is involved in the pathogenesis of several diseases. On the other hand, their biological activity may advantageously be used for the treatment of a variety of diseases, even if their administration may be associated with serious adverse effects. In this regard, a considerable individual variation in the therapeutic response and in the occurrence of side effects is well documented [1,2].

In the mammalian tissues, the prereceptor regulation of the GLCC action is dependent upon the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD, EC 1.1.1.146), which catalyzes the interconversion of biologically active GLCC (cortisol and corticosterone) and their inactive 11-keto forms (cortisone and 11-dehydrocorticosterone) [3]. In humans, two isoforms of this enzyme had been identified: type 2 (11β-HSD2) and type 1 (11β-HSD1) isozymes [4,5].

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doi:10.1016/j.jchromb.2005.11.011

11β-HSD2 is a high affinity nicotinamide adenine dinucleotide (NAD)-dependent unidirectional dehydrogenase, which exclusively converts cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, respectively. It is found mainly in mineralcorticoid target tissues such as kidney and colon, where it protects the mineralcorticoid receptor from cortisol excess. By contrast, 11β-HSD1, which is a nicotinamide adenine dinucleotide phosphate (NADP[H]) dependent bidirectional enzyme, functions predominantly in the reductase mode in intact cells and organs [6]. Its function is widely expressed in tissues, such as liver, lung, adipose tissue, vasculature, ovary, central nervous system and bone, where it may maintain high intracellular levels of the active form of GLCC [5,7]. Changes in the activity of 11β-HSD1 may be important in several human diseases, including hypertension, obesity, oligomenorrhea and hirsutism [8–13].

Modulation of 11β-HSD's activity, with the development of specific inhibitors, could offer a novel therapeutic approach. Moreover, as the therapeutic effectiveness of GLCC and the frequency of adverse events of the treatment are variable in different subjects, the study of this enzyme action could be important to understand the nature of this variability and to identify the subjects who may be more sensitive to the side effects of these substances.

In order to better understand the biological relevance of 11β-HSD in the pathogenesis of human diseases and in the variability of the response to treatment with GLCC, it is important to develop rapid assays for the evaluation of its activity in vivo.

Conventional assessments are based on measurements of the ratios of cortisol and cortisone and/or their metabolites, both free and/or conjugated. Very often the ratio tetrahydrocortisol (THF), allo-tetrahydrocortisol (A-THF) to tetrahydrocortisone (THE) in urine from 24 h collection is assayed (Fig. 1) [14,15].

Several analytical methods have been proposed in the literature for the determination of the ratio (*R*): (THF + A-THF)/THE. A large part of them used gas-chromatographic (GC) techniques including GC–MS [16–20], and required tedious and extensive extraction and derivatization steps.

Our target was to find an alternative approach to directly determine *R* in urine by using liquid chromatography coupled to tandem mass spectrometry with a minimal sample treatment. Such a direct determination needed to face some problems and pre-requisites related to the chemical structure and behavior of THF, THE, and steroids in general. In fact, urine is particularly rich of different steroidic components, only a small part of which is in the free form (urinary free steroids), being the major fraction constituted by glucuronide and sulphate conjugates [21]. Unfortunately, most fragment ions observed in their tandem mass spectra are common to different components, therefore a complete specificity is not possible. Moreover, steroid compounds tend to easily lose water molecules in the source, which further reduces the selectivity. For these reasons the HPLC method must be able to separate in a satisfactory way the components of interest. In addition, as the method intends to determine the total fraction of THF, A-THF, and THE, enzymatic hydrolysis of the conjugated species is advantageous. Of course, also this latter process needs to be investigated accurately in order to achieve a complete deconjugation. From the point of view of mass spectrometry, source parameters need to be optimized for the dehydrated forms, which provide precursor ions able to supply the best sensitivity in selected reaction monitoring (SRM) mode. Finally, due to the complicated nature of the matrix and possible suppression effects, the use of deuterated internal standard(s) is highly recommendable to assure an optimal accuracy.

The structure of the molecules used in this study is shown in Fig. 2.

2. Experimental

2.1. Solvents and reagents

All the solvents used (methanol, water and acetonitrile) were HPLC grade from Carlo Erba Reagenti S.p.A. (Rodano, MI, Italy), as well as ammonium acetate and formic acid. 5β-Pregnan-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol, THF), 5α-pregnan-3α,11β,17α,21-tetrol-20-one (allo-tetrahydrocortisol, A-THF), 5α-pregnan-3β,11β,17α,21-tetrol-20-one (3β,5α-tetrahydrocortisol, S-THF) and 5β-pregnan-3α,11α,17α,21-tetrol-20-one (epi-tetrahydrocortisol, E-THF) were purchased from Steraloids, Inc. (Newport, RI, USA); 3α,17α,21-trihydroxy-5β-pregnan-11,20-dione (tetrahydrocortisone, THF), tetrahydrocortisol-21-yl-β-D-glucuronide (THF-GLU) and tetrahydrocortisone-3α-yl-β-D-glucuronide (THE-GLU) were from Sigma–Aldrich Corporation (St. Louis, MO, USA); 5α-pregnan-11β,17α,21-triol-3,20-dione-1,2,4,5-D4 (allo-dihydrocortisol, D4-A-DHF) was obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Que., Canada); 5α-pregnane-3α,21-diol-20-one-17,21,21-D3 (D3-PGD) and 5α-pregnane-3α-ol-20-one-17,21,21,21-D4 (D4-PGO) were from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). β-Glucuronidase/arylsulfatase from *Helix Pomatia* (EC 3.2.1.31/EC 3.1.6.1) was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

2.2. Mass spectrometry

The mass spectra and the SRM determinations were obtained from an Applied Biosystems-MDS Sciex API 4000 triple quadrupole mass spectrometer (Concord, Ont., Canada), equipped with Turbo-V IonSpray (TIS) source. For the optimization, each compound was infused by a syringe pump Harvard Mod. 22 (Harvard Apparatus, Holliston, MA, USA).

The operative parameters resulted are as follows: ionspray voltage (IS), 5.5 kV; gas source 1 (GS1), 50; gas source 2 (GS2), 50; turbo temperature (TEM), 350 °C; entrance potential (EP), 10 V; declustering potential (DP), 60 V; scan range, as needed, depending on the analyte (expected protonated ion *m*/*z* value ±80 Th). MS–MS product ions were produced by collisional induced dissociation (CID) of selected precursor ions in the LINAC collision cell (Q2) and mass-analyzed in the second mass filter (Q3). Additional experimental conditions for MS–MS product ions spectra included collision (CAD) gas, nitrogen; CAD gas pressure, 2.9 mPa; collision energy (CE), 30 eV, collision cell exit potential (CXP), 15 V. SRM transitions

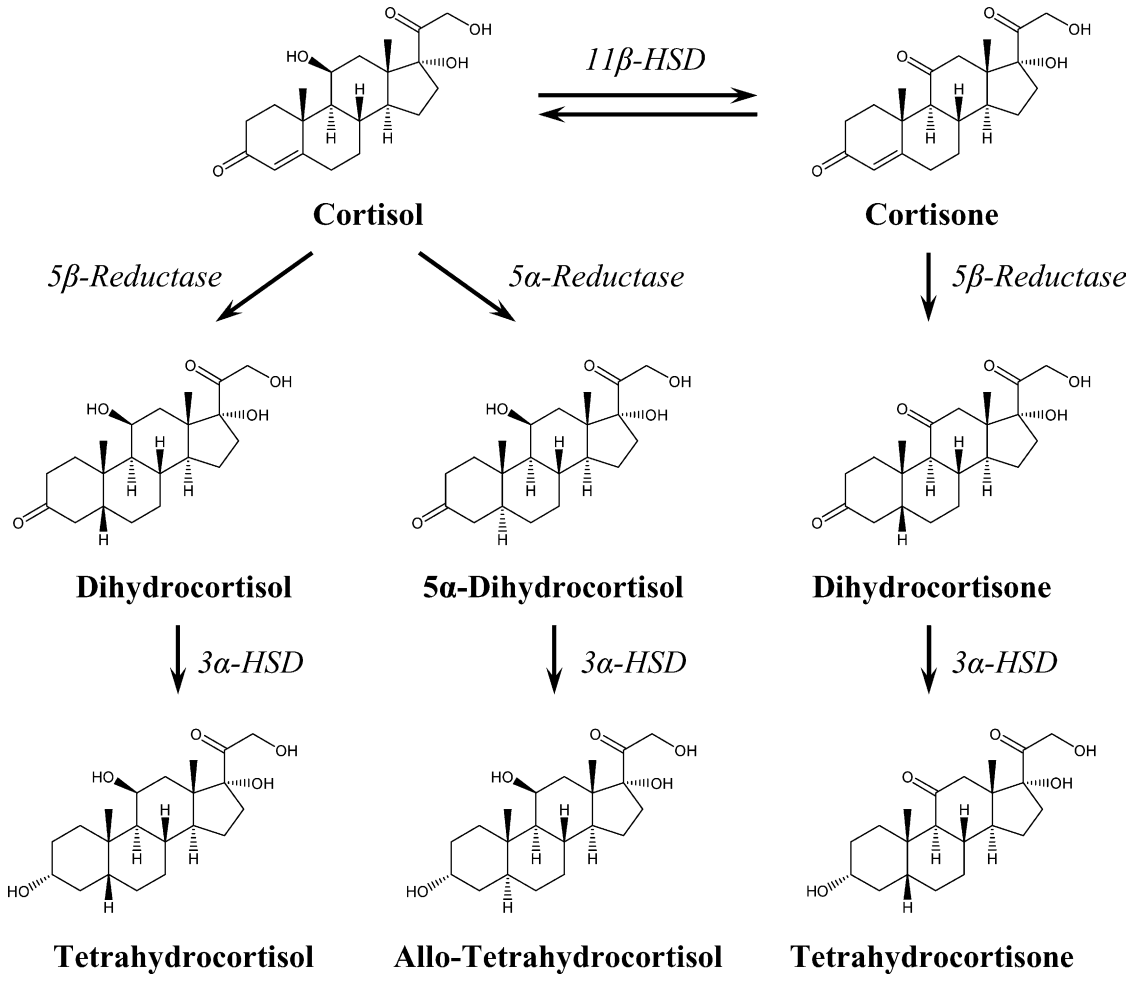


Fig. 1. Pathways of cortisol metabolism.

and the related optimized DP, CE and CXP for different analytes are shown in Table 1.

2.3. High performance liquid chromatography

HPLC separations were carried out by a Perkin-Elmer Series 200 Micro High pressure mixing pump equipped with a Series 200 Autosampler (Perkin-Elmer, Boston, MA, USA). Several columns and methods were tested. The final optimized method made use of a Phenomenex LUNA C8 2 mm × 50 mm, 3 μm particle size column (Phenomenex, Torrance, CA, USA) with the following mobile phase gradient:

Solvent A: Water/methanol 1:1 containing 0.1% formic acid.
Solvent B: Metanol/acetonitrile 1:1 containing 0.1% formic acid.
Gradient conditions: Five minutes from 100% A to 100% B; 3 min 100% B; 3 min equilibration time.

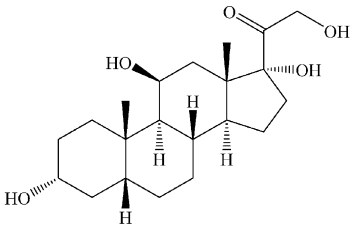
2.4. Sample preparation

A 10 ml aliquot was taken out of the 24 h excreted urine and centrifuged at 5000 rpm for 30 min. One milliliter of super-

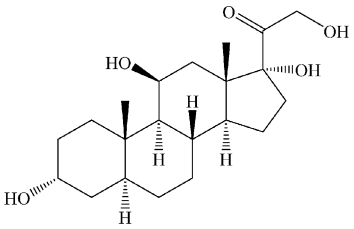
Table 1
SRM transitions and the related optimized source parameters for different analytes and labeled standards

Analytes	Operative parameters			
	SRM transition	DP	CE	CXP
THF (all)[a]	349 → 91	42	77	8
	349 → 105		62	10
THE	347 → 91	99	68	6
	347 → 149		33	12
THF-GLU	525 → 331	47	25	8
	525 → 349		15	9
THE-GLU	541 → 347	55	25	10
	541 → 365		14	10
D4-A-DHF	369 → 118	101	31	9
	369 → 229		31	6
D3-PGD	320 → 91	70	63	8
	320 → 135		26	11
D4-PGO	323 → 75	42	38	8
	323 → 135		29	12

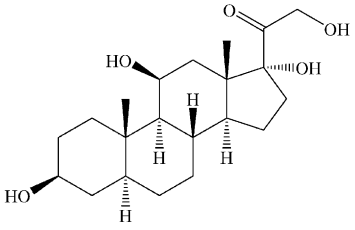
[a] The SRM transitions and the related optimized compound parameters are the same for all the THF isomers.



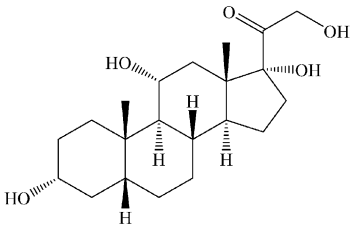
5β-pregnan-3α,11β,17α,21-tetrol-20-one
(Tetrahydrocortisol, THF)



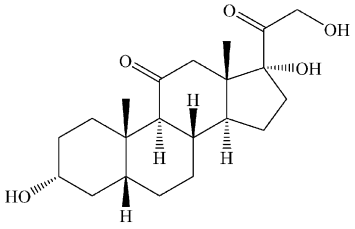
5α-pregnan-3α,11β,17α,21-tetrol-20-one
(Allo-Tetrahydrocortisol, A-THF)



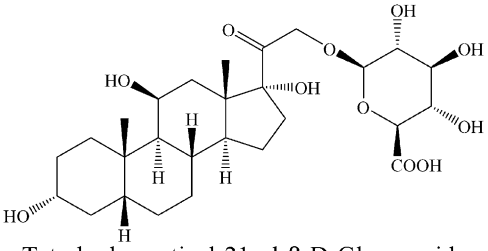
5α-pregnan-3β,11β,17α,21-tetrol-20-one
(3β,5α-Tetrahydrocortisol, S-THF)



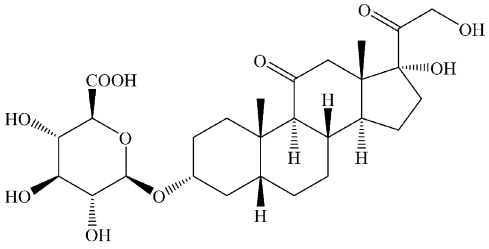
5β-pregnan-3α,11α,17α,21-tetrol-20-one
(Epi-Tetrahydrocortisol, E-THF)



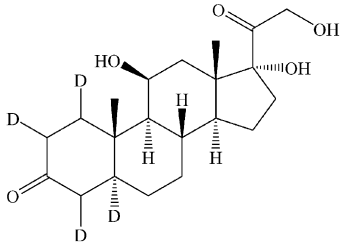
3α,17α,21-trihydroxy-5β-pregnan-11,20-dione
(Tetrahydrocortisone, THE)



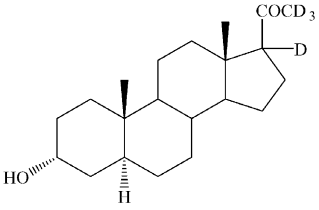
Tetrahydrocortisol-21-yl-β-D-Glucuronide
(THF-GLU)



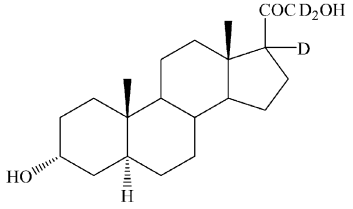
Tetrahydrocortisone-3α-yl-β-D-Glucuronide
(THE-GLU)



5α-pregnan-11β,17α,21-triol-3,20-dione-1,2,4,5-D4
(Allo-Dihydrocortisol, D4-A-DHF)



5α-pregnane-3α-ol-20-one-17,21,21,21-D4
(D4-PGO)



5α-pregnane-3α,21-diol-20-one-17,21,21-D3
(D3-PGD)

Fig. 2. Structures of the investigated compounds.

natant was placed in a 2 ml 12 × 32 vial together with 100 μl acetate buffer 1 M (pH 5.5), 20 μl β-glucuronidase/arylsulfatase [21,22], and a suitable amount of internal standard (20 μg of D3-PGD or D4-PGO or, alternatively, 10 μg of D4-A-DHF) dissolved in 20 μl of methanol. The mixture was incubated at 37 °C for 24 h, and then diluted 1:20 with pure water. As a consequence, the final internal standard concentration was 1000 ng/ml for D3-PGD and D4-PGO, and 500 ng/ml for D4-A-DHF. Twenty microliters of this final mixture was injected in the HPLC–MS–MS system in triplicate, as well as the standard solutions for the calibration curves.

2.5. Calibration curves

Seven standard solutions for the calibration curves were prepared containing 2.5, 5, 10, 25, 50, 100 and 250 ng/ml of both THF and A-THF, and 5, 10, 20, 50, 100, 200 and 500 ng/ml of THE. The internal standard concentration was the same for all the samples.

3. Results and discussion

As already stated in the introduction, THF and THE tend to lose water molecules even under soft ionization techniques such as ESI. Based on precedent experiences on similar molecules in our laboratory, this trend looks more related to thermal dehydration reactions prior to the ionization process than to in-source fragmentation. The MS spectra of THF and THE, reported in the Fig. 3, show that at least three water molecules are lost. The relative intensity of the four species ($[M+H]^+$, $[M–H_2O+H]^+$, $[M–2H_2O+H]^+$ and $[M–3H_2O+H]^+$) changes, depending on the source parameters. However, it is not possible to get a single ionic species clearly predominant with respect to the others. This feature represents a strong limit to the possibility to achieve a high sensitivity.

In addition, as shown by the spectra in Fig. 4, the fragmentation pattern of this class of compounds is quite complicated.

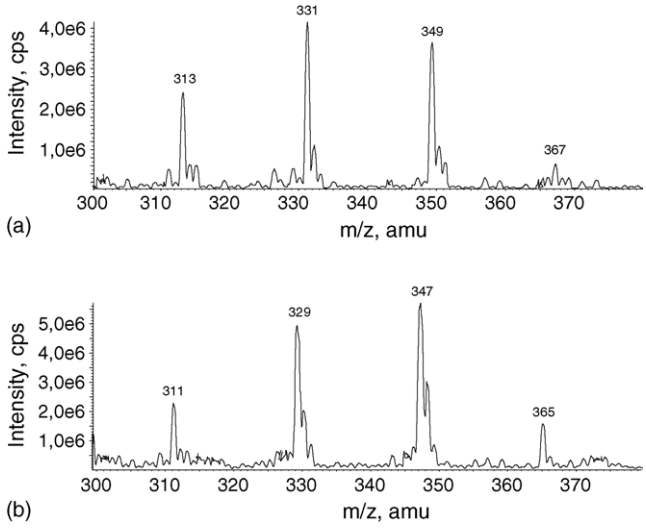


Fig. 3. ESI MS spectra of tetrahydrocortisol (a) and tetrahydrocortisone (b).

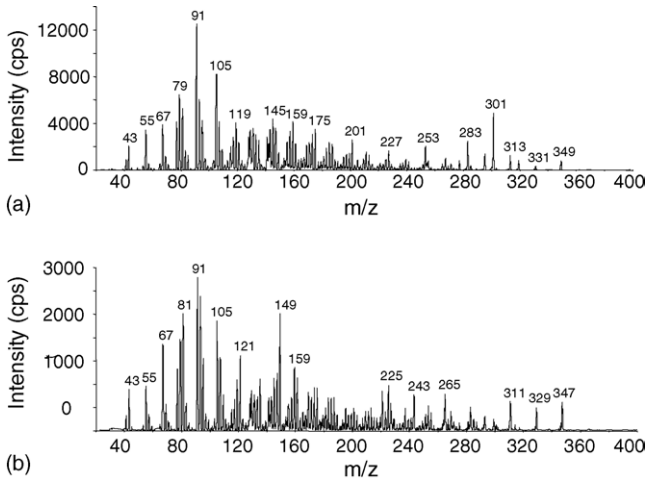


Fig. 4. MS–MS product ions spectra of tetrahydrocortisol (a) and tetrahydrocortisone (b).

Indeed, it gives rise to spectra containing a large number of fragments, which is similar to what can be observed in their electron ionization (EI) mass spectrum. Such a behavior causes the dispersion of the ionic current into a large number of fragment ions, only a limited number of which could be used for a SRM based method. It represents a further negative contribute to sensitivity, at least if compared to a situation where a single or a limited number of fragment ions are present in the spectrum. Moreover, most fragments are common to several steroid compounds, therefore the selectivity will not be as high as desirable. As a consequence, a good chromatographic separation is necessary to support the MS method.

Table 1 summarizes the SRM transitions selected and the relative optimized compound-dependant parameters for THF and THE. Under such conditions, the lowest concentration detected in pure water resulted to be 0.5 ng/ml for both compounds (S/N 10 and 18 for THF and THE, respectively). Experiments carried out on several batches of urine samples confirmed that the normal urine concentration of these analytes is relatively high. Such a high sensitivity is not strictly necessary. Nevertheless, an ultimate high sensitivity was aimed to allow a minimal sample preparation, which was merely dilution of the samples prior to the injection.

As far as the HPLC method is concerned, the separation of the two steroids, at the beginning, was carried out by a Discovery C18 2.1 × 50 mm, 3 μm particle size column (Supelco Inc., Bellefonte, PA, USA), together with the following mobile phase composition and elution program: 1 min 95% water + formic acid 0.1% (A)/5% acetonitrile + formic acid 0.1% (B); 6 min to 100% B, 1 min 100% B. Under these conditions the elution times of THF and THE in water were 4.84 and 4.98 min, respectively. However, when this method was used to run real urine samples from healthy volunteers, diluted 1:10 with pure water, two intense peaks at 4.73 and 4.82 min were present in the chromatograms. The relative SRM transitions were the same with respect to THF and THE standards. For this reason, we initially attributed these peaks just to THF and THE, with a retention time shift induced by some matrix effects. In contrast, their basal

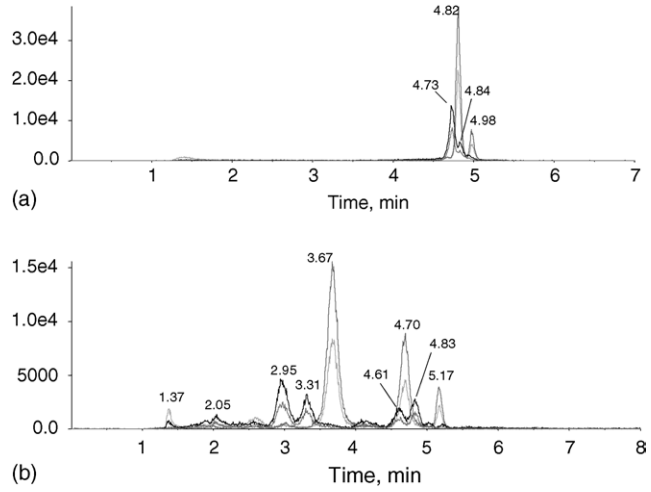


Fig. 5. Comparison of chromatographic separation of a real urine sample: initial HPLC conditions (a) vs. optimized method (b). The peaks at 4.84 (a) and 4.61 and 4.83 (b) are attributable to A-THF and THF (co-eluting in chromatogram a), and the peaks at 4.98 (a) and 5.17 (b) belong to THE.

amounts seemed to be too high in absence of cortisone based drug treatments. Anyway, when these samples were spiked with THF and THE standards, their chromatograms showed up four peaks, THF and THE eluting at the expected elution times of 4.84 and 4.98 min. It is worthy to note that under atmospheric pressure chemical ionization (APCI) THF and THE were detectable even at low concentrations (in water, 2 ng/ml with S/N 9 and 25, respectively), while the strong peaks at 4.73 and 4.82 min were not visible at all.

These experiments showed hat these latter signals could be attributed to interfering compounds, whose presence prevents the accurate integration of the species of interest. Hence, it was necessary to improve the separation, so that the origin of these interfering compounds could be identified.

Several HPLC columns and mobile phase combinations have been tested in order to achieve a better separation. Finally, the method described in the experimental part was able to separate the above interference peaks in several ones, all with the same fragmentation and ratio as THF and THE standards. As shown in Fig. 5, the two peaks observed in urine samples under the preliminary HPLC conditions spread out in at least four peaks for THF and three peaks for THE. This makes the quantitation possible, but we still wished to identify the origin of such interferences.

As stated in Section 1, urine is a matrix containing several steroid compounds, and most of them are in conjugated form, both sulphates and glucuronides. In our opinion the possible interfering compounds could be both THF and THE isomers and conjugates. For this reason, we checked different suppliers of laboratory chemicals, and we found three isomers of THF, and one THF and one THE glucuronides (see Fig. 2). Moreover, considering the opportunity of using an opportune stable isotope internal standard, we tested three commercial deuterated compounds with chemical structure similar to the compounds under investigation. The MS operative parameters relative to these species are summarized in Table 1, and their retention times, acquired by using the final HPLC method, are reported in Table 2. Under these HPLC conditions, a satisfactory separation was achieved among different analytes. Among the compounds having same transitions, only THF and A-THF showed poor resolution. This is not a problem, in our case, as they must be taken together for calculating the ratio *R*. It must also be noted that all together the chromatographic pattern of non-deuterated standards is very similar to that of real urine samples, indicating that our opinion was correct. Taking also into consideration that THF-GLU and THE-GLU show a very high response factor to ESI and no response at all to APCI, we can conclude that the severe interferences observed in real samples are originated from THF and THE isomers in conjugated form.

Table 2
Elution times of the different THF isomers, THE, glucuronide standards, and deuterated steroids used in the present investigation

Analytes	Elution time (min)
S-THF	3.40
THE-GLU	4.06
D4-A-DHF	4.13
A-THF	4.29
THF	4.39
THE	4.49
E-THF	4.57
THF-GLU	4.59
D3-PGD	5.32
D4-PGO	5.91

This means that in order to quantitate the total amount THF, A-THF, and THE, the urine samples must be at least treated to hydrolyze both glucuronides and sulfate steroids. Hence, we tested a treatment with a commercial mixture of β-glucuridase and arylsulfatase enzymes, and we found out that in a few hours (at 37 °C, see Section 2), the severe interferences described above completely disappeared, confirming that they are definitely due to presence of conjugate species. Fig. 6 shows the qualitative effect of the enzymatic hydrolysis on one among the THE glucuronides. In the final method, the hydrolysis time has been set to 24 h just to assure a complete reaction. This is the only manipulation needed, as, after the enzymatic deconjugation, the amounts present are big enough to allow a relatively high dilution of the urine sample (≥1:20). Both D4-A-DHF and D3-PGD (mostly the former) were considered as suitable internal standards, because their elution times are closer with respect to D4-PGO to the elution times of THF, A-THF, and THE, which is a good point to consider possible matrix effects. A good advantage in this case is the fact that we only need to determine a ratio of two analytes, i.e., the absolute amount of them is not of strong interest. A check of the matrix effect was performed, anyway, using the method proposed by King and co-workers [23] by post-infusion of a standard THF and THE mixture (100 ng/ml) in-line with an HPLC–MS–MS injection of a blank urine sample. The analytes and the selected internal standards elute in a region where the suppression effect is limited. As stated in Section 2, we repeated this experiment using different batches of urine samples.

The final method was tested on some batches of urine samples collected within 24 h from supposed healthy volunteers. Some

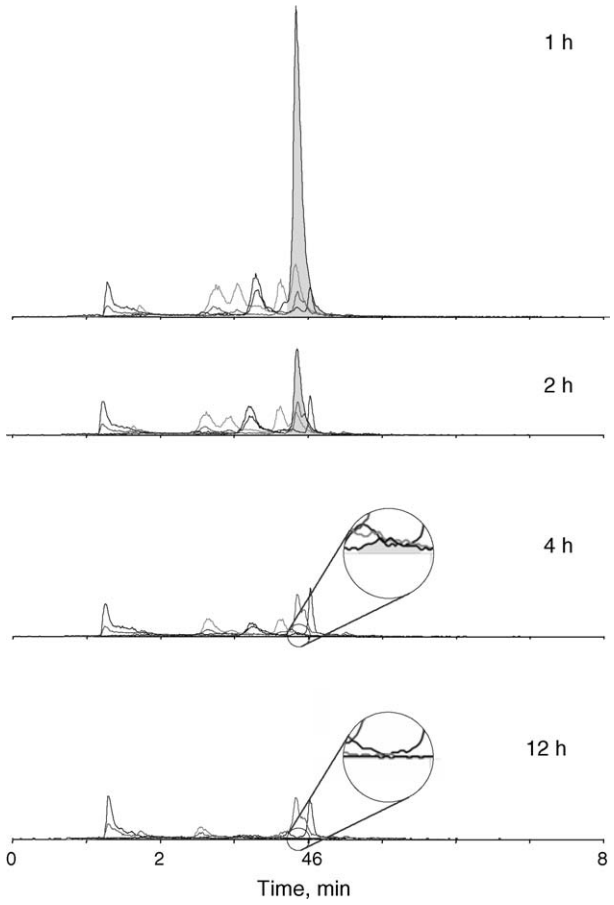


Fig. 6. Qualitative effect of the incubation time (1, 2, 4, and 12 h) on the enzymatic hydrolysis of THE glucuronide.

results, obtained from 13 samples analyzed using D4-A-DHF as internal standard, are summarized in Fig. 7, which shows that *R* ranges from 1.18 to 1.94, with a standard deviation constantly below 0.1 indicated by the error bars on the histogram. Thus, *R* is 1.45 on average, which is in agreement with reported values [24], even if very variable data can be found in the literature. Analyses carried out on batches containing numerous samples, using D3-PGD as internal standard, gave equivalent results.

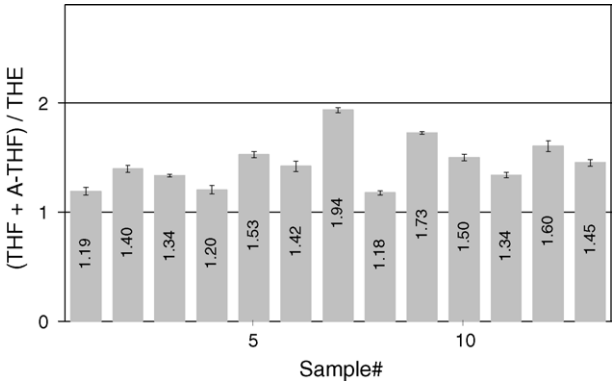


Fig. 7. Graphic representation of the results relative to 13 different urine samples from 24 h collection. Each sample was analyzed in triplicate.

4. Conclusions

The results presented here give once more a clear indication of the utility of liquid chromatography coupled to tandem mass spectrometry for the analysis of complex matrices, and in particular for the assay of clinically interesting parameters in biological fluids, such as the ratio (THF + A-THF)/THE. They also provide a further example of the challenging development of an analytical method when severe matrix effects are present. Only an accurate optimization of both MS and HPLC separation conditions, together with a deep investigation on the nature of interfering species and the use of deuterated internal standards, eventually allow a good quantitation.

The method proposed here is not intended to be a definitive one, suitable for high-throughput analysis. Some work is still necessary in order to improve both reliability and ruggedness. However, it nevertheless represents a good starting point for setting up an ultimate rapid method for the monitoring of such a highly diagnostic ratio on routine bases. At present, experiments directed towards a further optimization of the enzymatic hydrolysis and a prevention of the progressive HPLC column performance degradation, are in progress in our laboratory.

Anyway, the preliminary results obtained are in a good agreement with data available in the literature, which further support the validity of this approach.

Acknowledgement

The authors wish to thank Prof. Giancarlo Saba for helpful discussions.

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