Quantification of DHEA in human serum on a routine basis: development and validation of a tandem mass spectrometry based method

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ABSTRACT

In the clinical laboratories dehydroepiandrostenedione (DHEA) is usually quantified by immunoassay based methods, which are often affected by cross-reactivity with endogenous interferences, such as 4-androsten-3β-ol-17-one. The interfering compounds lead to a poor accuracy of the measurements, mainly at a low concentrations level. The present paper describes a validated method based on tandem mass spectrometry coupled to liquid chromatography, for the accurate quantification of DHEA in serum, which provided good results in the assessment of DHEA in plasma, too. The peculiarity of this method is the use of calibrators and quality controls prepared by adding measured amounts of DHEA-D_5, a stable isotope labeled analogue of DHEA, to real serum from healthy subjects. DHEA-D_5 was used in place of DHEA, which is usually present in unstripped serum at physiological levels, as it has the same basic structure, provides an equivalent instrumental response, and can be easily distinguish by DHEA by mass spectrometry due to its different m/z value. The method proved to be sensitive, with a LLOD of 0.34 ng/ml and a LLOQ of 1.18 ng/ml, and selective, with overall performances that allow its use on a routine basis.

Keywords: DHEA, DHEA-D_5, laboratory medicine, liquid chromatography, tandem mass spectrometry.

INTRODUCTION

DHEA (dehydroepiandrostenedione) is a multi-functional endogenous steroid hormone [1]. It is the most abundant circulating steroid hormone in young adult humans and its levels change during the different stages of humans life, in particular it declines with age and during certain types of illness or stress [2]. DHEA is the precursor of sex steroid hormones, both androgens and estrogens, and it is synthetized in the andrenal glands (80%), in the male and female gonads, and in the brain [3]. However, DHEA is an active hormone which is involved in a broad range of biological effects in humans and rodents. Studies focused on the identification of possible specific receptors binding DHEA as a ligand have been carried out for over 20 years [4]. A few of them demonstrated the existence of proteins in liver having this function, although the biochemical identity of these proteins has remained unknown [5,6]. In the last 10 years the biochemical role of DHEA and its effects in humans have been investigated. Furthermore, the mechanism of action of a series of orphan receptors, namely peroxisome proliferators activated receptor (PPAR), pregnane X receptor (PXR), constitutive androstanol receptor (CAR), and estrogen receptor β [7,8,9,10] have been elucidated and provided the evidence that DHEA and some of its metabolites either bind to or activate these newly characterized receptors [11,12].

DHEA belongs to androgens, which is a significant subclass of steroid hormones, both adrenal and gonadal, exerting their biological effects acting by genomic and nongenomic mechanisms [13]. In fact, androgens and their precursor DHEA bind specific cytosolic receptor, that, in common with other members of the nuclear receptor superfamily, acts as a ligand-inducible transcription factor. The binding of androgens to the androgens receptor (AR) in the cytoplasm [14] induces receptor dimerization, and the so formed dimer is then translocated into the nucleus where it regulates gene expression [15]. In addition to this transcriptional or genomic mode of action by steroids, it was suggested that androgens and DHEA, like progesterone and estrogen, may exert rapid, nongenomic effects [16]. Nongenomic steroid activity typically involves cell surface receptors and a rapid induction of conventional second messenger signal transduction cascades, including increases in free

intracellular calcium, and activation of protein kinase A (PKA), protein kinase C (PKC), and Mitogen-Activated Protein Kinases (MAPK) [15].Through these mechanism and as a precursor of androgens and estrogens, DHEA is involved and controls the development and the maintenance of male sexual phenotype, including embryo life differentiation of penis, scrotum and prostate, activity of the primary male sex organs (spermatogenesis), development of male secondary sex characteristics [17], ovarian activity, and the normal follicle maturation, i.e. it is involved in female fertility[18]. A derangement of DHEA and consequently of androgenic and estrogenic hormone levels in man and women in the different stage of life may lead to the development of several reproductive, metabolic, cardiovascular and neoplastic diseases [19]. As a consequence, quantification and monitoring of DHEA in biological fluids is important in pediatric, adult, geriatric endocrinology, as well as oncology [20].

For the above mentioned reasons, there is a considerable interest in the androgens monitoring and in a reliable and practical measurement of low DHEA serum concentrations. Nowadays, in the clinical laboratories, DHEA in biological fluids is mainly quantified by immunoassay, although it will increasingly be replaced by mass spectrometry based methods, such as tandem mass spectrometry coupled to liquid chromatography [21]. Actually, immunoassay tests are often affected by cross-reactivity with endogenous interferences, which could induce an overestimate of the effective concentrations, mainly at a low concentrations levels. As demonstrated by a proficiency testing (PT) carried out by the College of American Pathologists (CAP), most laboratories involved in steroid analysis use several different immunoassay (IA) based methods, and this poor standardization among all of them reflects in a limited accord in the laboratory results. The main differences in the results from different laboratories have to be attributed to the use of different antibodies, having a different specificity with respect to the target analytes [22]. In contrast, CAP itself demonstrated a better agreement among steroid measurement from those laboratories using LC-MS-MS. Due to that, mass spectrometry, usually LC–MS-MS, is emerging as one of the most promising approaches for the monitoring of endocrine and metabolic diseases [23]. Compared to the traditional techniques,

advantages of tandem mass spectrometry include enhanced specificity, accuracy, precision, sensibility, ability to simultaneously measure multiple analytes in highly complex biological matrices, and a reduced amount of sample needed for analysis, thereby enhancing diagnostic capabilities and enabling a better patient care. As a consequence, at present LC-MS-MS is considered as the technique of choice for the accurate quantification of endogenous steroids in biological specimens [24].

At first, our interest in DHEA was mainly focused to the safety and the activity of DHEA in combination with an aromatase inhibitor (AI) in hormone receptors positive and triple negative advanced breast cancer patients, where AI was used to prevent any possible conversion of DHEA to estrogens. This investigation, which was a not-randomized phase II study, needed an accurate method to assay DHEA in plasma and serum samples, which was developed on purpose.

Hence, in the present paper, we describe a reliable, sensitive and accurate HPLC-MS-MS based validated method to assay dehydroepiandrosterone (DHEA) in serum, which was developed for clinical research and that is presently used for clinical analysis on a routine basis. Its accuracy is largely attributable to a novel calibration procedure, which makes use of real serum matrices from humans, i.e. not submitted to any preliminary manipulation such as steroid removal and lyophilization, for the preparation of the standard working solutions used to build the calibration curves. The method was validated for serum samples and tested for plasma samples, too.

MATERIALS AND METHOD

Reagents and materials

Dehydroepiandrosterone-D_5 (DHEA-D_5), 17-hydroxyprogesterone-D_8 (17-OHP-D_8), used as internal standard (IS) were provided by Sigma-Aldrich (Saint Louis, MO, USA), as well as acetonitrile (LC-MS grade), methanol (LC-MS grade), ultra-pure water (LC-MS grade), ammonium acetate solution 7.5M (CH_3COONH_4, for molecular biology), and formic acid (MS grade ≈ 98%).

Instrumentation

All the analyses were performed by an instrument layout consisting of an AB Sciex API 4000 triple quadrupole mass spectrometer (Concord, ON, Canada), equipped with an APCI source, a quaternary HPLC pump (Series 200, PerkinElmer, Boston, MA, USA), and an Agilent 1290 Infinity UHPLC system (Santa Clara, CA, USA), constituted by an autosampler outfitted with peltier tray, a binary pump, and a column oven. A ten port switching valve (Valco Instruments Co. Inc., Huston, TX, USA) was used as a divert valve. Chromatographic separation was performed by a Phenomenex (Torrance, CA, USA) Luna 3 µm C8 (2) 100 Å, 50 x 3 mm HPLC column protected by a C8 4 x 2.0 mm ID security guard cartridge. Data acquisition and system control were carried out by an AB Sciex Analyst® version 1.6.3 software, while data processing by an AB Sciex Multiquant® version 3.0.2 software.

Sample collection, storage and preparation

Venous blood samples were collected by venipuncture in tubes with no clot activator. The whole bloods were left undisturbed at room temperature for as long as necessary to get a good coagulation and then were centrifuged at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge. Supernatants were moved to empty tubes and stored at -20 °C until processing. When needed, they were thawed at room temperature, vortexed (15 sec) and a 150 µl aliquot was added with 300 µl of a freshly prepared DPS (Daily Precipitation Solution) containing acetonitrile, formic acid 0.1% (V%) and IS (0.59 ng/ml). The obtained suspensions were vortexed (15 min) and centrifuged (18,620 x g, 15 min). 300 µl of supernatants were then collected and dried under a gentle stream of nitrogen at 40° C. The dried residues were reconstituted with 125 µl of a mixture of the HPLC eluents, solvent A and solvent B (35/65; V/V), whose composition will be described in the following “HPLC conditions” section, and the reconstituted matters were vortexed (15 min) and 60 µl of the obtained solutions were injected into the LC-MS-MS system.

Preparation of standard stock solution, calibration curve and QC

The solutions of DHEA-D_5 (100 µg/ml) and 17-OHP-D_8 (100 µg/ml) were stored at -20°C and used as standard stock solutions. The calibration curve of DHEA-D_5 was built using seven standard solutions (0.34 (L1), 0.85 (L2), 2.12 (L3), 5.30 (L4), 13.24 (L5), 33.11 (L6), and 82.76 ng/ml (L7)), each of them prepared by serial dilution in unstripped serum. Three quality controls, prepared by adding known amounts of DHEA-D_5 solution to unstripped serum in order to get 0.74 ng/ml (QC1), 3.68 ng/ml (QC2), and 25.74 ng/ml (QC3) concentration levels, were analyzed in each batch. Both calibration standards and QC samples (150 µl each) were added with DPS and treated like the samples.

HPLC conditions

The HPLC separation was carried out by the Agilent binary pump, by using CH_3COONH_4 in methanol 1.2 mM, as a solvent A, and CH_3COONH_4 in a mixture water/methanol (98/2, V/V) 1.2 mM, as a solvent B, under the gradient conditions shown in table 1. The chromatographic column was kept at 40 °C. In order to ensure to the analytical method an adequate robustness and preventing a progressive degradation of the instrumental sensitivity, the divert valve was properly set in order to discard both head and tail of the HPLC runs. In practice, the mass spectrometer was supplied with the column eluate in the time range 7.5 ÷ 9.3 min and with methanol and water from the PerkinElmer quaternary pump for the remaining run time. The injection volume of samples, QCs and calibrators was 60 µl.

Mass spectrometry conditions

The MS method was based on positive ion mode selected reaction monitoring (SRM). Using optimized declustering potential (DPs), collision energies (CEs), and collision exit potentials (CXPs), two transitions were monitored for each compound, the more intense which, was used as a quantifier (Q), while the other one as a qualifier (q). These parameters are listed in table 2. Further operative

parameters were set as follows: nebulizer current (NC), 3.0 KV; gas source 1 (GS1) zero air, 55; source temperature (TEM), 600°C; entrance potential (EP), 9.4 V; IQ1 lens potential, -10 V; collision gas (CAD) nitrogen, operative pressure with CAD gas on, 5.0 mPa.

Method validation

To ensure data reliability, reproducibility and robustness, the method described above has been validated with reference to sensitivity, selectivity, accuracy, precision, reproducibility and stability, in compliance with EMA guideline [25]. Matrix effect was studied as well.

Selectivity is defined as the ability of analytical method to measure and differentiate the analyte and the IS from other components in the sample, behaving as potential interfering compounds. It was evaluated by injecting structurally similar compound, such as 4-androsten-3β-ol-17-one (4-DHEA), 17α-hydroxyprogesterone (17-OHP), and testosterone, into the HPLC-MS-MS device and running the system by the method under development. The standard solutions containing the above mentioned compounds, separately or in a mixture, were at a concentration level of 40 ng/ml (4-DHEA) and 20 ng/ml (17-OHP and testosterone).

The **Linearity** of an analytical procedure is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample. So that the method linearity was evaluated within the calibration curve range and it was tested from 0.34 ng/ml through 82.76 ng/ml.

The **accuracy** of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage), while the **precision** describes the closeness of repeated individual measures of analyte and it is expressed as relative standard deviation (RSD%). Intra-day accuracy and precision were evaluated by analyzing five human spiked QC samples with DHEA-D_5, at three different concentrations levels: 0.74 ng/ml (QC1), 3.68 ng/ml (QC2), 25.74 ng/ml (QC3). Inter-day accuracy and precision were evaluated by three replicates analyses for QC1, QC2 and QC3 in three different days.

Lower limit of detection (LLOD) and **lower limit of quantification** (LLOQ) were calculated on the criterion of Signal to Noise ratio S/N 3 and 10, respectively, and they were evaluated by analyzing human spiked samples with DHEA-D_5 at different concentration levels. The signal to noise ratio was obtained by a software tool included in the Analyst® version 1.6.3 package.

Recovery is a measure of the efficiency at which an analytical method recovers the analyte through the sample preparation. Recovery was evaluated by spiking a real serum sample with DHEA-D_5 at three different concentration levels , i.e QC1, QC2 and QC3, and it was calculated comparing detected concentrations of DHEA-D_5 added before (A.B.) and after (A.A.) protein precipitation, in compliance with the following formula:

$$R\% = (A.B./ A.A.) \cdot 100$$

The **stability** evaluation shows how storage conditions, sample preparation and sample analysis affect the analyte concentration. It was evaluated by checking the storage conditions and by performing analysis of DHEA, DHEA-D_5 and 17-OHP-D_8 stock solutions, as well as serum samples spiked with DHEA-D_5 at three concentration levels (QC1, QC2 and QC3). The stability of serum spiked samples was tested by comparing freshly prepared samples and the same samples after a freeze/thaw cycle.

Matrix effect was qualitatively investigated by post column infusion experiments, by an apparatus constituted by the HPLC system, a Harvard Apparatus Model 22 (South Natick, MA,USA) syringe pump, and a tee connector to mix the flows from the two pumps before the delivery to the mass spectrometer [26]. Eluate from the HPLC column, containing the matrix components, was delivered at a 600 mL/min flow rate, while DHEA and 17-OHP-D_8 aqueous solutions, 1000 ng/ml and 100 ng/ml respectively, were infused at a 20 µl/min flow rate by the syringe pump. Real samples previously submitted to sample preparation or water were injected by the autosampler into the HPLC column and the infusion response was monitored [26]. Matrix effect was evaluated in several human serum matrices.

RESULTS AND DISCUSSION

Calibrators an quality controls

DHEA-D_5, a stable isotope labeled analogue of DHEA which perfectly mimics it and provides an equivalent instrumental response, was used instead of DHEA for the preparation of calibrators and QCs in human serum. In fact, real serum contains endogenous DHEA at physiological levels and cannot be used as it is as a blank matrix for the preparation of standard solutions. It should be submitted to stripping of the steroidal compounds by active charcoal prior the addiction of measured amounts of DHEA. However, charcoal is an unselective adsorbent able to remove steroids as well as a large number of other chemical species having affinity for it, deeply affecting the serum matrix composition, which when treated results very different from the real samples. On the contrary, DHEA-D_5 in an authentic matrix behaves like the endogenous DHEA in human samples and makes calibrators and QCs very similar to real samples, with significant advantages in terms of method performances. By using this procedure, the calibration curves for DHEA assessment were built by using seven standard solutions in real human serum containing DHEA-D_5 at the concentration levels of 0.34 (L1), 0.85 (L2), 2.12 (L3), 5.30 (L4), 13.24 (L5), 33.11 (L6), and 82.76 ng/ml (L7), where 0.34 ng/ml corresponds to the lower limit of detection (LLOD) of the analytical method. Also three quality controls (QC) were prepared by the same procedure, at the following concentration levels: 0.74, 3.68, and 25.74 ng/ml.

DHEA extraction from serum

A sample preparation preceding the analysis is usually carried out in order to reduce the complexity of the sample matrices and to make samples more suitable for chromatographic separation and mass spectrometry detection. In our method a simple protein precipitation by a acetonitrile added with formic acid 0.1% (V%), mixed to the sample in the proportion 1:3 (sample:acetonitrile, V:V), was satisfactory and moreover not very time consuming. Actually, time consumption of the sample preparation step is a key point for assays used on routine bases because it has a significant influence

on both rates and throughput. Hence, this point has to be taken into serious account during the method design.

Optimization of HPLC-MS-MS

The development of the Mass Spectrometry SRM (Selected Reaction Monitoring) based method started with the evaluation of two different ion sources, ESI (ElectroSpray Ionization) and APCI (Atmospheric Pressure Ionization), which were tested using the optimal instrumental parameters, tuned up in order to achieve the best possible performances for both sources. APCI demonstrated to be the best option in terms of ionization efficiency and chemical background, and this is in accordance with many data from the literature for steroid hormones [27]. Independently on the instrumental settings, even under a soft ionization technique such as ESI and APCI, steroids often loose water molecules in the ion source [28]. In particular, during APCI ionization, DHEA and DHEA-D_5 lost water molecules, showing dehydrated pseudo-molecular ions on the kind of $[M+H-H_2O]^+$ at m/z 271.2 Da and 276.2 Da, respectively, which were suitable as precursor ions in the SRM method. On the contrary, 17-OHP-D_8, used as an internal standard, was less responsive to dehydration reactions and provided a $[M+H]^+$ ion at m/z 339.3 Da, intense enough to be used as a precursor ion. Several product ions from each precursor ion were monitored and just two transitions were selected, one as a qualifier and the other one as a quantifier. Product ions were chosen according to their structural representativity, stability, intensity and signal-to-noise ratio in real samples. Chromatography was set up in order to separate the analyte of interest from interfering compounds having a similar structure, which could affect the measurements, and to ensure a method ruggedness and a good selectivity. A 60 µl injection of reconstituted sample, QC or calibrator was considered as the most suitable because it provided good results in terms of signal intensity, peak shape, and column lifetime; so that it was used as a standard injection volume in the method. A 100 ul injection was also tested with several hundreds of samples and provided an optimal method sensitivity, particularly advantageous when DHEA at a very concentration level

have to be assayed. Unfortunately such a large amount of sample severely affected the column lifetime, allowing no more than 400 injections, less than one half of the usual column lifetime.

Method validation

The final method was submitted to internal validation, and the outcomes are reported below. The retention times (t_r) recorded for DHEA, DHEA-D_5, 17-OHP-D_8 were 8.64±0.06, 8.62±0.06, 8.57±0.05 min (mean±SD), respectively. Calibration curve showed a good linearity in the tested range with a reproducible slope and correlation coefficient (R), always greater than 0.998. The linear regression provided y = mx+q as a curve equation, with m = 0.0340±0.0070 and q = -0.0039±0.0035, obtained using a weighting of 1/x. LLOD and LLOQ were 0.34 ng/ml and 1.18 ng/ml respectively. The DHEA-D_5 recovery rates were largely dependent on the concentration value and were 65%, 62% and 91% for, 0.74 ng/ml (QC1), 3.68 ng/ml (QC2), 25.74 ng/ml (QC3), respectively. These values were identical to those found for DHEA and that is in a good agreement with our expectations. The intra- and inter-day accuracy (accuracy %) and precision (RSD %) were acceptable at all QC concentration levels, the accuracy always was in the range 85-115% and precision was satisfactory, with a RSD% < 15%. These results are summarized in table 3. The stability test pointed out that the stock solutions of DHEA, DHEA-D_5 and 17-OHP-D_8 in methanol are stable at least two years at -20 °C and the quantified concentrations of frozen-thawed (1 cycle) samples were 92%, 95%, 95% of the initial value respectively for QC1, QC2, QC3. Post infusion experiments, carried out by infusing either DHEA or 17-OHP-D_8 and eluting some different real serum samples, demonstrated that matrix effect was quite constant in the time range 1.5 ÷ 10.2 min and no significant suppression effect affected our analyte, as well as IS. In fact, SRM chromatograms in the figure 1 are quite flat and show just a strong peak at t_R 5.25 min, attributable to DHEAS, a steroid compound structurally similar to DHEA which responds to the same SRM transitions used for DHEA, i.e. 271.2 → 197.2 and 271.2 → 213.2. The presence of DHEA's potential interfering compounds was checked. Primarily, the HPLC separation between DHEA and 4-DHEA was proved, in order to ensure that no overlapping

of their chromatographic peaks was present. This is crucial because these two compounds respond to the same SRM transitions and cannot be separated by mass spectrometry, due to the significant similarity between their structures. In addition, both chromatographic and mass spectrometry behaviors of other similar steroids, such as testosterone and 17-OHP, were checked. Actually, 17-OHP coeluted with DHEA, but tandem mass spectrometry was selective enough to distinguish these two steroids. On the contrary, there was no coelution of testosterone and DHEA, although their retention times were close to each other and, as a consequence, no reciprocal interference occurred.

DHEA assay in human serum

The analysis of sera confirmed the suitability of the method for the quantification of DHEA. About 1,000 samples, either from healthy volunteers or from patients were assayed. Each daily batch included two sets of calibrators and QCs, injected one at the beginning of the sample list and the other one at the end. This was carried out in order to check the inter-batch stability, which usually was excellent, save where instrumental problems occurred. Acquired data have been processed by Multiquant® software, which allowed the quantification of endogenous DHEA by using the calibration curves built with DHEA-D_5. An example of the SRM chromatograms acquired by our method is shown in figure 2, where chromatograms from two samples and one calibrator in the same acquisition batch are reported: (a) L5 calibrator containing DHEA-D_5 at a 13.24 ng/ml concentration level (central peak, 8.67 min) and endogenous DHEA from the human serum used as a matrix (right peak, 8.69 min); (b) endogenous DHEA in a human serum sample at a normal concentration level (right peak, 8.69 min); (c) endogenous DHEA at an abnormal high concentration level (peak at 8.69 min) of 730 ng/ml, whose value was out of the calibration range and required a 10-folds sample dilution before the protein precipitation step [29]. In addition, all of the SRM chromatograms in the figure exhibit also the internal standard peak at 8.60 min, except for chromatogram (c), where it was about negligible with respect to DHEA.

Conclusions

We present here a simple and efficient LC-MS-MS method to assay DHEA, which was developed and validated for blood serum, the sample fluid usually collected in the hospitals for steroid hormone analysis on routine bases, and was tested with about one thousand samples. The method provided good results also with plasma, which was usually collected for some research purposes, such as the breast cancer project previously mentioned. Our results demonstrated its reliability, sensitivity, reproducibility, and accuracy, as well as its compatibility with clinical diagnostic on routine basis. This method is a further evidence of the significant contribution of tandem mass spectrometry to clinical chemistry and laboratory medicine. Unfortunately we had not the possibility to introduce the simultaneous analyses of multiple targeted steroid hormones from a single sample. It would allow a better use of the mass spectrometry potential, providing, at the same time, a complete set of information to be used for the diagnosis and treatment of diseases. However, nowadays the admittance of mass spectrometry in a conventional clinical laboratory requires compromises and changes which must necessarily be progressive and that will take time to achieve the optimal use of this technique and make possible its integration with the traditional technologies.

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Table 1. Program of HPLC pumps.

Step	HPLC Quaternary Pump				HPLC Binary Pump			
	Total time (min)	Flow rate (µl/min)	Methanol (%)	Water (%)	Total time (min)	Flow rate (µl/min)	Solvent A (%)	Solvent B (%)
0	0.0	200	50	50	0.0	600	35	65
1	11.7	200	50	50	3.0	600	35	65
2					4.0	600	45	55
3					9.0	600	65	35
4					9.1	600	100	0
5					11.5	600	100	0
6					11.6	600	35	65
7					11.7	600	35	65

Table 2. Mass Spectrometry operative parameters

		Operative Parameters		
Analyte	SRM transition	DP	CE	CXP
DHEA	271.2 → 197.2 (q) 271.2 → 213.2 (Q)	57	27 23	4.9 5.3
DHEA-D_5	276.2 → 202.2 (q) 276.2 → 218.2 (Q)	57	27 23	4.9 5.3
17-OHP-D_8	339.2 → 100.1 (Q) 339.2 → 113.1 (q)	76	44 33	8.0 9.7

Table 3. Accuracy and precision for DHEA-D_5.

Intra-day variation

	Mean	*RSD (%)*	*Accuracy (%)*
QC1	1.06	8.55	106.17
QC2	5.17	6.24	103.45
QC3	32.22	3.90	92.06

Inter-day variation

	Mean	*RSD (%)*	*Accuracy (%)*
QC1	0.95	8.12	94.82
QC2	4.20	4.28	84.04
QC3	34.24	3.15	97.85

Figure legends

Figure 1. Post column infusion experiment to monitor matrix effect exerted by (a) water on DHEA, (b) human serum from a healthy volunteer on DHEA, (c) water on 17-OHP-D_8, and (d) human serum on 17-OHP-D_8. The dashed lines indicate the theoretical retention times of 17-OHP-D_8 (8.60 min) and DHEA (8.69 min), pointing out that no significant suppression or enhancement effects affected the signal of both analyte and internal standard.

Figure 2. Quantitative SRM transitions chromatograms from the L5 calibrator (a), a sample with a DHEA concentration of 6.17 ng/ml (b), and a sample with a very high DHEA concentration level, measured at 730 ng/ml (c).

Fig. 1

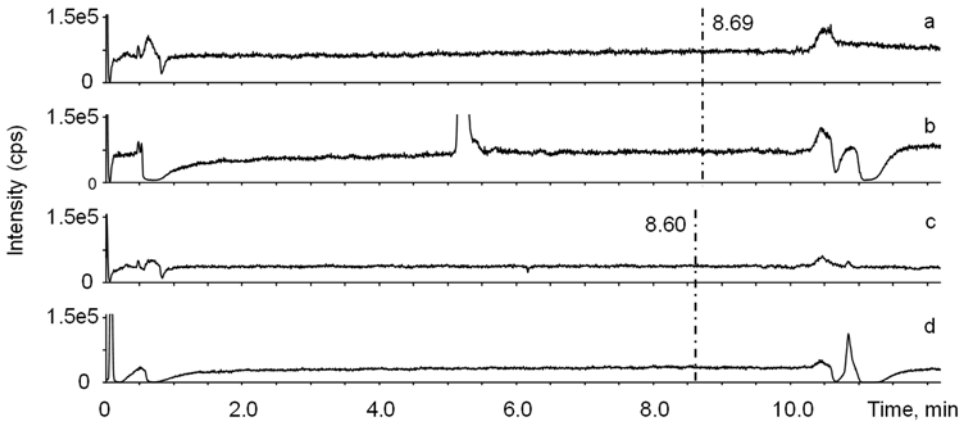
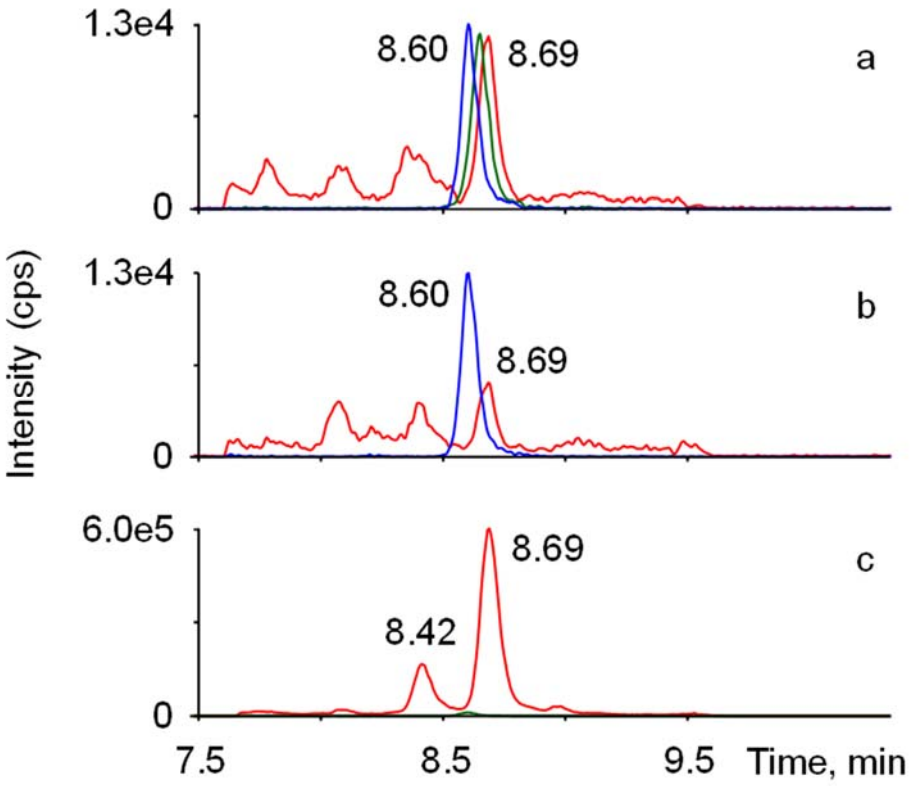


Fig. 2


1.3e4
8.60
8.69
a
0
1.3e4
8.60
b
8.69
0
Intensity (cps)
6.0e5
8.69
c
8.42
0
7.5
8.5
9.5
Time, min