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Development and validation of a high-performance liquid chromatography assay for the quantitative determination of 7-oxo-dehydroepiandrosterone-3β-sulfate in human plasma

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Abstract

A new, simple, reproducible and reliable high-performance liquid chromatography method with ultraviolet absorbance detection at 240 nm was developed and validated for the determination of 7-oxo-dehydroepiandrosterone-3β-sulfate in human plasma. The method was based upon solid-phase (C_{18}) extraction of plasma after addition of 17β-hydroxy-3β-methoxy-androst-5-en-7-one as internal standard. Using 1 ml of plasma for extraction, the detection limit of the assay was 3 ng/ml. The standard curve was linear over the concentration range 10–1000 ng/ml. Stored at −20°C for about 4 months at various concentrations in plasma, 7-oxo-dehydroepiandrosterone-3β-sulfate did not reveal any appreciable degradation. Also included herein is a method for the simultaneous detection and determination of 7-oxo-dehydroepiandrosterone and 7-oxo-dehydroepiandrosterone-3β-acetate in plasma.

Keywords: 7-Oxo-dehydroepiandrosterone-3β-sulfate; 7-Oxo-dehydroepiandrosterone; 7-Oxo-dehydroepiandrosterone-3β-acetate

1. Introduction

7-Oxo-dehydroepiandrosterone (II, 7-oxo-DHEA, Fig. 1) is produced metabolically from dehydroepiandrosterone (DHEA, I) in several different tissues [1–4]. 7-Oxo-DHEA (II) is more effective than DHEA (I) (a) in inducing thermogenic enzymes [5,6], (b) in production of interleukin-2 by isolated T cells [7], (c) in reversal of memory-abolition by scopolamine [8], and (d) in enhancement of memory in old mice [8]. Because neither DHEA (I) nor its 7-oxo derivative is a ligand for a specific receptor but both have striking effects when administered to animals, we presume that they are on a metabolic pathway from cholesterol to one or more cellularly active hormones.

7-Oxo-DHEA (II), unlike DHEA (I) is not converted to either androgens or estrogens [5]. It is not harmful to rats or monkeys when administered at 1000 mg/kg body weight [9] or to humans when administered at 200 mg daily for 4 weeks [10].

7-Oxo-DHEA was isolated from human urine [11] and peripheral venous plasma [12] more than three

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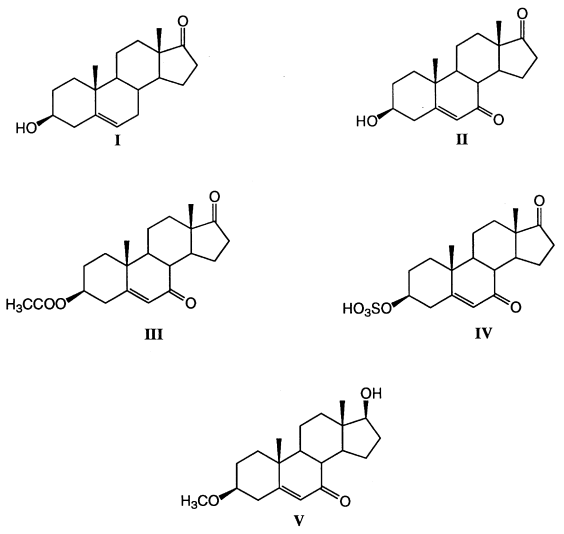

Fig. 1. Structures of (I) dehydroepiandrosterone, (II) 7-oxo-dehydroepiandrosterone, (III) 7-oxo-dehydroepiandrosterone-3β-acetate, (IV) 7-oxo-dehydroepiandrosterone-3β-sulfate, (V) 17β-hydroxy-3β-methoxyandrost-5-en-7-one (I.S.).

decades ago but no serious attempts have been made to develop and validate its methods of analysis in biological fluids or tissues. A gas chromatography–mass spectrometry method for the analysis of 7-oxo-DHEA-3β-sulfate from the blood of a patient with paraquat poisoning has been reported [13].

To the best of our knowledge, no HPLC method has been reported in the literature for the determination of 7-oxo-DHEA (II) or its conjugates in biological fluids.

The present HPLC method was developed to study the behavior of 7-oxo-DHEA (II) and/or its major metabolites in human volunteers who had been administered 7-oxo-DHEA as its acetyl derivative (7-oxo-DHEA-3β-acetate, III). The 3-acetyl group is added to protect the compound against oxidation during synthesis, but is readily hydrolyzed in vivo, as are short chain esters of other steroids [6,14].

2. Experimental

2.1. Materials and reagents

7-Oxo-DHEA (II), 7-oxo-DHEA-3β-acetate (III), 7-oxo-DHEA-3β-sulfate (IV) and 17β-hydroxy-3β-methoxyandrost-5-en-7-one (V, the internal standard) were synthesized in the laboratory, and their purity checked by HPLC (>99.5%). Stock solutions (1 mg/ml) were prepared in methanol and stored at −5°C. Methanol, acetonitrile, diethyl ether and tetrabutylammonium dihydrogenphosphate (Fluka brand ion pair reagent) were purchased from Aldrich and used as such. Phosphoric acid, HPLC grade (Fisher brand) was used to adjust the pH of the aqueous phase. Distilled water, deionized and purified by Nanopure ultra pure water system from Barnstead (USA) was used (18.25±0.05 MΩ cm). Sep-Pak C_{18} cartridges (3 ml capacity) from Waters Assoc. (Milford, MA, USA) were used for sample preparation. Hydrophilic PTFE syringe filters (Millex-LH, 0.5μm) were purchased from Millipore, and tubes with teflon lined screw caps (Pyrex brand) were silanized and used for extraction.

Pooled plasma samples from healthy human volunteers, obtained from University of Wisconsin–Madison Hospital, were used for the validation of the method.

Statistical calculations were performed with the help of SYSTAT 7.0 software, supplied by the SPSS Inc. USA.

2.2. Instrumentation

The chromatographic system consisted of a Hewlett-Packard 1100 series HPLC system, comprised of a quaternary pump (G1311A) with on line degasser, thermostated column compartment (G1316A), autosampler (G1313A) and a diode array UV detector (G1315A). Data were acquired and processed using HP Chemstation version A.05.01 software from Hewlett-Packard.

2.3. Chromatographic conditions

Chromatography was performed on a Zorbax C_{18} analytical column (150×4.6 mm I.D.) packed with 3.5 μm diameter particles (Eclipse-XDB, Mac Mod). The analytical column was protected using a C_{18} guard column (Supelguard, LC-18 DB, 20×4.6 mm I.D. cartridge, Supelco). The flow-rate was set at 1.0 ml/min and the eluent was monitored at 240 nm with a 4 nm band width. The reference wavelength was set at 360 nm for 7-oxo-DHEA-3β-sulfate (IV)

and 390 nm for 7-oxo-DHEA (II) and 7-oxo-DHEA-3β-acetate (III) with a bandwidth of 100 nm. The slit width was kept at 4 nm. The injected volume was 75 μl. The column temperature was maintained at 40.0±0.5° C. The mobile phase consisted of 5.0 m*M* tetrabutylammonium dihydrogenphosphate (TBADHP, adjusted to pH 3.40 with 1 *M* phosphoric acid), acetonitrile and methanol. All solvents were filtered (0.45 μm) before use. A gradient elution was used. For 7-oxo-DHEA-3β-sulfate (IV), the mobile phase composition was acetonitrile–methanol–5.0 m*M* TBADHP (30:10:60, v/v) at time $t=0$, (35:10:55) at $t=15$, (75:10:15) at $t=17$ to 18 and (30:10:60) i.e. the initial composition at $t=20$ (System A). For 7-oxo-DHEA (II) and 7-oxo-DHEA-3β-acetate (III), a faster gradient system was used. The mobile phase composition was: acetonitrile–methanol–5m*M* TBADHP (35:10:55, v/v) at time $t=0$, (60:10:30) at $t=10$, (80:10:10) at $t=12$ and (35:10:55) i.e. the initial composition at $t=15$ (System B). A 15-min post run time was used to re-equilibrate the column and for the baseline to return to the normal.

2.4. Extraction procedure

2.4.1. 7-Oxo-DHEA-3β-sulfate (IV)

The internal standard (80 ng in 20 μl methanol) was added to 1.0 ml of plasma in a 5-ml polypropylene tube followed by 1.0 ml of water. The contents were vortex mixed for 10 s, centrifuged for 3 min at 1000 *g* and the supernatant was carefully transferred to a Sep-Pak (C_{18}) cartridge, which had previously been washed with methanol (5 ml) and water (5 ml). The loaded cartridge was washed with 10 ml water and the desired compounds were eluted with methanol (3 ml). Methanol was carefully evaporated to dryness at 40°C under a slow stream of nitrogen. The residue was dissolved in the mobile phase of initial concentration (150 μl), filtered through a 0.5-μm filter and a 75-μl aliquot was injected into the chromatograph.

2.4.2. 7-Oxo-DHEA (II) and 7-oxo-DHEA-3β-acetate (III)

The internal standard (80 ng in 20 μl methanol) was added to 1.0 ml of plasma in a 5-ml polypropylene tube followed by 1.0 ml of water. The contents were vortex mixed for 10 s, centrifuged for 3 min at 1000 *g* and the supernatant was carefully transferred to a Sep-Pak cartridge, which had previously been washed with methanol (5 ml) and water (5 ml). The cartridge was washed with 10 ml water and 2 ml 20% methanol. The desired compounds were eluted with methanol (3.0 ml), transferred into a 15-ml screw capped tube and carefully evaporated at 40°C under a slow stream of nitrogen. The residue was dissolved in diethyl ether (5.0 ml) by vortex mixing (2×10 s), water (5 ml) was added and the contents were vortex mixed thoroughly (30 s). The tubes were closed and centrifuged for 3 min at 1000 *g*. The ether layer was separated by snap drying the aqueous layer in an acetone–dry ice bath and evaporated to dryness under a slow stream of nitrogen. The residue was dissolved in mobile phase of initial concentration (150 μl), filtered (0.5 μm) and a 75-μl aliquot was injected into the chromatograph.

2.5. Standard curve

The standard stock solutions were prepared by dissolving separately 25 mg of 7-oxo-DHEA-3β-sulfate (IV) and the internal standard (V) in 25 ml of methanol. From these stock solutions, working solutions were prepared at final concentrations of 10, 20, 50, 100, 500 and 1000 ng/20 μl of 7-oxo-DHEA-3β-sulfate (IV) containing 80 ng/20 μl of the internal standard. All solutions were stored at −5°C until use.

Calibration standards were prepared using concentrations of 10, 20, 50, 100, 500 and 1000 ng/ml of 7-oxo-DHEA-3β-sulfate (IV) in human plasma and water. The standard samples were prepared by adding appropriate concentrations of 7-oxo-DHEA-3β-sulfate (IV). The volume added was always smaller than or equal to 2% of the total volume of the sample, so that the integrity of the sample was maintained. These samples were processed as described earlier.

2.6. Stability study

In the present study, the stability of 7-oxo-DHEA-3β-sulfate (IV) in plasma was assessed from spiked samples (20, 100 and 500 ng/ml), after bench top

storage at room temperature for 4 h, at −20°C for 4 months, and after subjecting them to repeated freeze–thaw cycles. The samples were analyzed immediately after preparation and after storage as per the protocol. Prior to the analysis of the samples after cold storage, they were brought to room temperature (20°C) and vortex mixed well. Each determination was performed in replicate ($n=5$).

2.7. Data analysis

The ratio of peak area of the compound of interest to that of internal standard was used for the calculations and assay. Peak-area ratios were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares regression analysis. The linearity of the method was confirmed using the classical statistical tests, i.e. correlation coefficients and comparison of intercept with zero.

2.8. Recovery

The recovery of 7-oxo-DHEA-3β-sulfate (IV) was determined by comparing peak area ratios from plasma spiked with known amounts of 7-oxo-DHEA-3β-sulfate (10, 20, 50, 100, 500 and 1000 ng/ml), processed as described earlier versus peak area ratios of the same concentrations extracted from water.

The absolute recovery of 7-oxo-DHEA-3β-sulfate (IV) was determined by comparing peak area ratios from plasma spiked with known amounts of 7-oxo-DHEA-3β-sulfate (10, 20, 50, 100, 500 and 1000 ng/ml), processed as described earlier versus peak area ratios of the same concentrations injected directly into the chromatograph. Each sample was analyzed five times.

2.9. Accuracy, precision and limit of quantitation

The intra-day precision and accuracy of the method were evaluated by analyzing, on the same day, five replicates of spiked samples at four different concentrations (20, 50, 100 and 500 ng/ml) against a calibration curve. The inter-day precision and accuracy were assessed by analyzing spiked quality control samples at four different concentrations on different days ($n=8–24$) against a calibration curve.

Accuracy was evaluated as percentage error (mean of measured-mean of added)/mean of added. The precision was given by inter-day and intra-day coefficient of variation.

The linearity of the method was assessed from 10 to 1000 ng/ml of 7-oxo-DHEA-3β-sulfate (IV). Each concentration was measured five times.

The limit of detection was defined as the sample concentration of 7-oxo-DHEA-3β-sulfate resulting in a peak height of three times S/N.

The limit of quantitation was defined as the sample concentration of 7-oxo-DHEA-3β-sulfate resulting in a peak height of ten times S/N.

2.10. Specificity

To evaluate the specificity of the method, plasma samples were subjected to the assay procedure and the retention times of endogenous substances in plasma were compared with those of compounds of interest. Interference from the internal standard on the retention time of 7-oxo-dehydroepiandrosterone-3-sulfate and vice versa was checked to rule out the presence of any interfering impurities.

Specificity of the method was assessed to test matrix influence between different plasma samples from various healthy human volunteers ($n=24$).

3. Results and discussion

3.1. HPLC of 7-oxo-DHEA-3β-acetate (III)), 7-oxo-DHEA (II) and 7-oxo-DHEA-3β-sulfate (IV)

Since dehydroepiandrosterone (I) is known to exist predominantly in blood as its sulfate conjugate, the initial experiments were devoted to learn the fate of 7-oxo-DHEA-3β-acetate (III) in plasma. 7-Oxo-DHEA (II), 7-oxo-DHEA-3β-acetate (III) and 17β-hydroxy-3β-methoxyandrost-5-en-7-one (V, internal standard) were well resolved using a gradient elution system consisting of acetonitrile, methanol and 5 m*M* tetrabutylammmonium dihydrogenphosphate as mentioned earlier. As absorbance maxima of 7-oxo-DHEA (II) and related compounds were observed to be close to 240 nm, in the present study this wavelength was selected. A satisfactory resolution of 7-oxo-DHEA (II), 7-oxo-DHEA-3β-acetate (III) and

the internal standard (V) was achieved by gradient elution on a 15-cm reversed-phase column at a reference wavelength of 390 nm (band width 100 nm). The overall run-time, including the post run time for re-equilibration of the column, for the chromatogram was 35 min.

Under the present chromatographic conditions, 7-oxo-DHEA-3β-acetate (III) was not detected in the human plasma after a single dose of 100 mg p.o. in eight human volunteers. 7-Oxo-DHEA (II) was however detected in trace quantities, but the concentration was too low (<5 ng/ml) to permit quantitation by this method. The representative chromatograms are shown in Fig. 2. However significant amounts of 7-oxo-DHEA-3β-sulfate (IV) were detected in the subjects' plasma. Hence HPLC conditions were developed for the estimation of 7-oxo-DHEA-3β-sulfate (IV) in human plasma.

7-Oxo-DHEA-3β-sulfate (IV) was also resolved using a gradient elution system consisting of acetonitrile, methanol and 5 m*M* tetrabutylammonium dihydrogenphosphate. The representative chromatograms are shown in Fig. 3. The effect of reference wavelength and band-width was systematically studied and the values giving best results in terms of baseline noise were selected. No interfering peaks at the retention times of 7-oxo-DHEA-3β-sulfate (IV) and internal standard were detected. The mean retention times, observed during a span of about 1 month, were 12.16±0.15 min (CV=1.21%, n=17) for 7-oxo-DHEA-3β-sulfate (IV) and 10.35±0.05 min (CV=0.47%, n=17) for the internal standard.

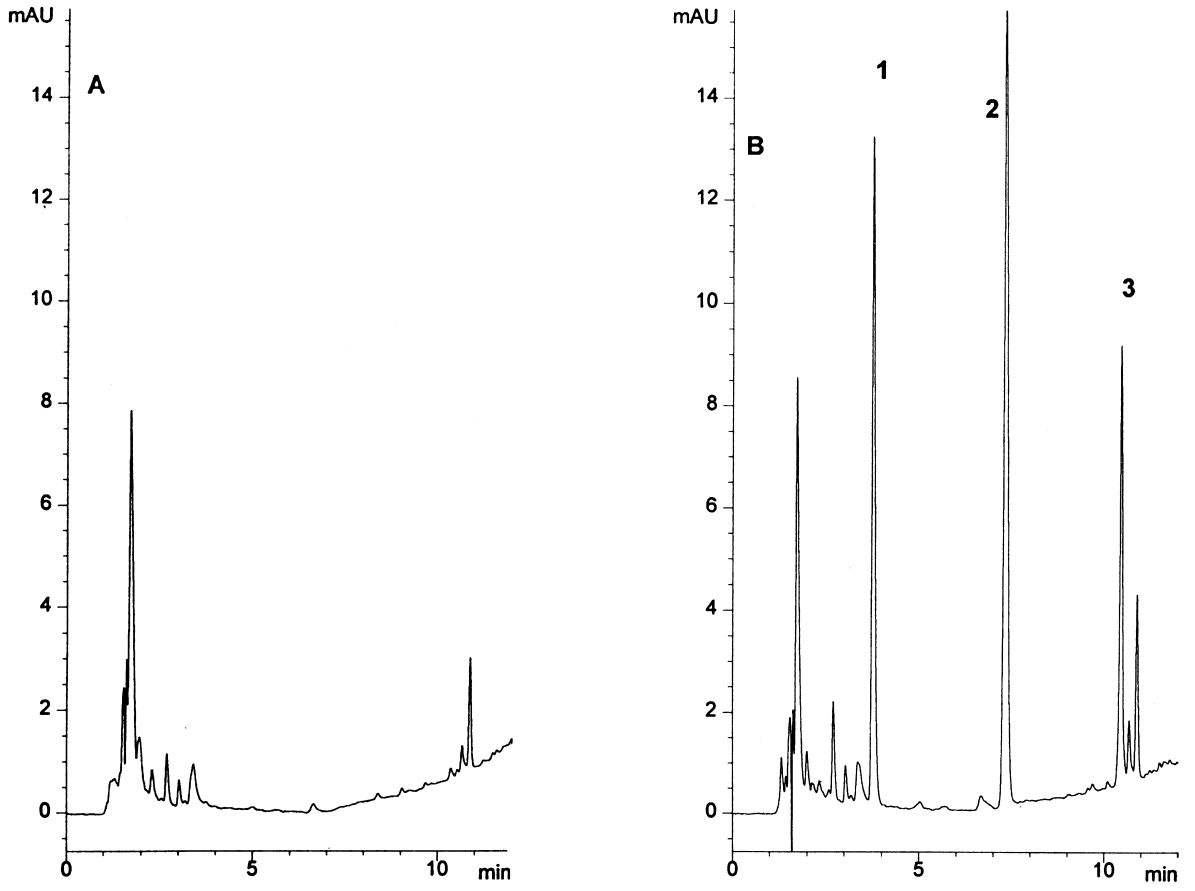

Fig. 2. Chromatograms of (A) blank human plasma, (B) human plasma spiked with 50 ng/ml of 7-oxo-DHEA, 50 ng/ml of 7-oxo-DHEA-3β-acetate and 100 ng/ml of I.S. Peaks: 1=7-oxo-DHEA; 2=I.S.; 3=7-oxo-DHEA-3β-acetate. Chromatographic conditions as mentioned in Section 2.3, mobile phase: System B.

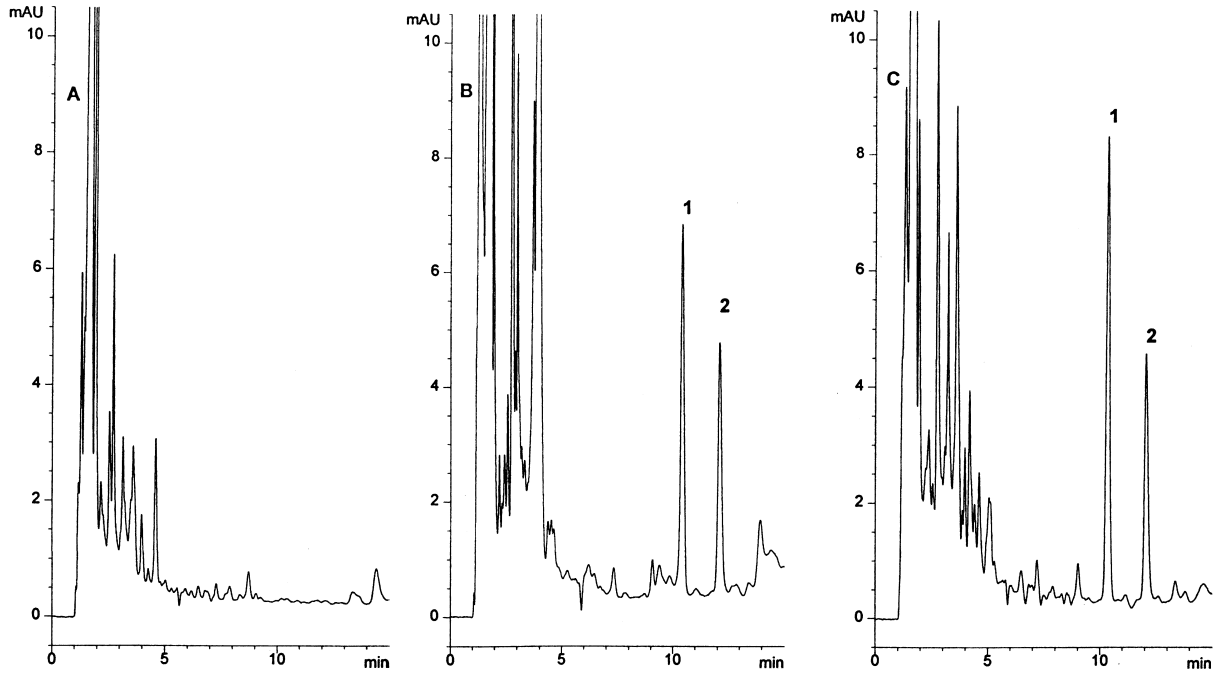

Fig. 3. Chromatograms of (A) blank human plasma, (B) human plasma spiked with 100 ng/ml of 7-oxo-DHEA-3β-sulfate and 80 ng/ml of I.S. and (C) subject dosed with 100 mg 7-oxo-DHEA-3β-acetate (120-min sample), concentration of 7-oxo-DHEA-3β-sulfate, 81.4 ng/ml. Peaks: 1=I.S.; 2=7-oxo-DHEA-3β-sulfate. Chromatographic conditions as mentioned in Section 2.3, mobile phase: System A.

The effectiveness of both solid-phase extraction (SPE) and liquid–liquid extraction of 7-oxo-DHEA (II), 7-oxo-DHEA-3β-acetate (III) and 7-oxo-DHEA-3β-sulfate (IV) were carefully studied. All the three compounds of interest as well as internal standard could be successfully eluted from the C_{18} cartridge with methanol. 7-Oxo-DHEA-3β-sulfate (IV) was successfully resolved from plasma peaks after the solid-phase extraction. However in the case of 7-oxo-DHEA (II) and 7-oxo-DHEA-3β-acetate (III), some minor interferences from plasma necessitated further clean-up of the sample for the chromatography. This was achieved by evaporating the methanol under nitrogen, dissolving the residue in diethyl ether followed by washing of the ether layer with water.

As a whole, the extraction procedures developed for the isolation of 7-oxo-DHEA (II) and related compounds from plasma proved satisfactory in terms of reproducibility of the operation, percentage recovery and purity of the extracts for the subsequent chromatographic analysis.

The performance of the column was closely monitored during the study period. The resolution between 7-oxo-DHEA-3β-sulfate (IV) and the internal standard (V) was 6.55±0.37 (CV=5.61%, n=17).

The complete study (>1500 biological samples), including preliminary studies carried out to develop the current method, was conducted on the same column.

3.2. Linearity

The response of the area ratios of the various concentrations (10, 20, 50, 100, 500 and 1000 ng) of 7-oxo-DHEA-3β-sulfate (IV) were studied and found to be linear when plotted against actual quantities. The peak area ratios for six different concentrations of 7-oxo-DHEA-3β-sulfate (IV), extracted from water were plotted against the added quantities.

In plasma the peak-area ratio of 7-oxo-DHEA-3β-

sulfate (IV) and the internal standard varied linearly with concentration over the 10–1000 ng/ml range. The correlation coefficients for the calibration curves were equal to or better than 0.999.

The slope of the fitted straight line (10–1000 ng/ml) for the calibration curve prepared on the same day using the same stock solution was 0.0067 ml/ng, the intercept being −0.0362. The corresponding coefficient of the linear regression analysis was 0.9992.

3.3. Limit of quantitation and limit of detection

The limit of quantitation was 10 ng/ml, the corresponding coefficient of variation ($n=5$) was 7.1%. At this level, the percentage error was found to be 6.8%.

The limit of detection was 3 ng/ml. A representative chromatogram is shown in Fig. 4.

3.4. Extraction recovery

The mean extraction recovery of 7-oxo-DHEA-3β-sulfate (IV) was satisfactory, mean±SD= 82.2±4.4 (CV=5.4%), range 74%–86%, and was consistent in the range of assay.

3.5. Accuracy and precision

The coefficient of variation for within run precision, calculated from replicate ($n=5$) determinations of the same plasma was 5.9% for 20 ng/ml, 2.6% for 50 ng/ml, 0.8% for 100 ng/ml and 2.0% for 500 ng/ml. The corresponding values for between-run precision were 16.2, 12.9, 10.5 and 7.5%, respectively. The mean recovery for 7-oxo-DHEA-3β-sulfate observed during within-run experiment was 100% for 20 ng/ml, 97.6% for 50 ng/ml, 103.7% for 100 ng/ml and 106.0% for 500 ng/ml concentration. The percentage error ranged from 0.0 to 6.0%. The percentage recovery for between-run analysis ranged from 94.0 to 99.5% with a percentage error of 0.5 to 6.0%.

The results of accuracy and precision [within-run precision (repeatability) and between-run] for the analysis of 7-oxo-DHEA-3β-sulfate (IV) are given in Table 1.

3.6. Specificity

No endogenous substance interfered at the retention time of 7-oxo-DHEA-3β-sulfate (IV). There was no interference from the 7-oxo-DHEA-3β-sulfate on the retention time of internal standard and vice versa. 7-Oxo-DHEA (II) as well as 7-oxo-DHEA-3β-acetate (III) did not interfere with the peak of 7-oxo-DHEA-3β-sulfate (IV). From random plasma samples ($n=24$ volunteers), no interference of the matrix was observed. However the plasma obtained from UW hospital exhibited more baseline noise in comparison to that observed with volunteers' plasma.

3.7. Stability

7-Oxo-DHEA-3β-sulfate (IV) was stable in plasma for 4 h at room temperature (percent recoveries 100–102%) and for 4 months at −20°C (percent recoveries 103–112%). No significant deviation (CV 4% or less) was found from the nominal values. At least three freeze–thaw cycles can be tolerated without losses higher than 12% (CV <10%). The values are given in Table 2.

3.8. Clinical studies

The method developed was found to be suitable for the analysis of samples collected during clinical investigations (single dose as well as steady state) in humans. It has been used for the determination of 7-oxo-DHEA-3β-sulfate (IV) in plasma samples of twenty-two volunteers.

4. Conclusions

Since 7-oxo-DHEA (II) is a metabolite of DHEA (I) and the latter predominantly exists as its sulfate conjugate in blood during its short sojourn in the body, it was considered logical to expect the same for the 7-oxo-DHEA (II). We were unable to detect 7-oxo-DHEA-3β-acetate (III) (the form in which 7-oxo-DHEA was administered) in the blood plasma of eight volunteers. Moreover only a very small amount of free 7-oxo-DHEA (II, ~ 5 ng/ml or less) was observed in the plasma. The majority of 7-oxo-

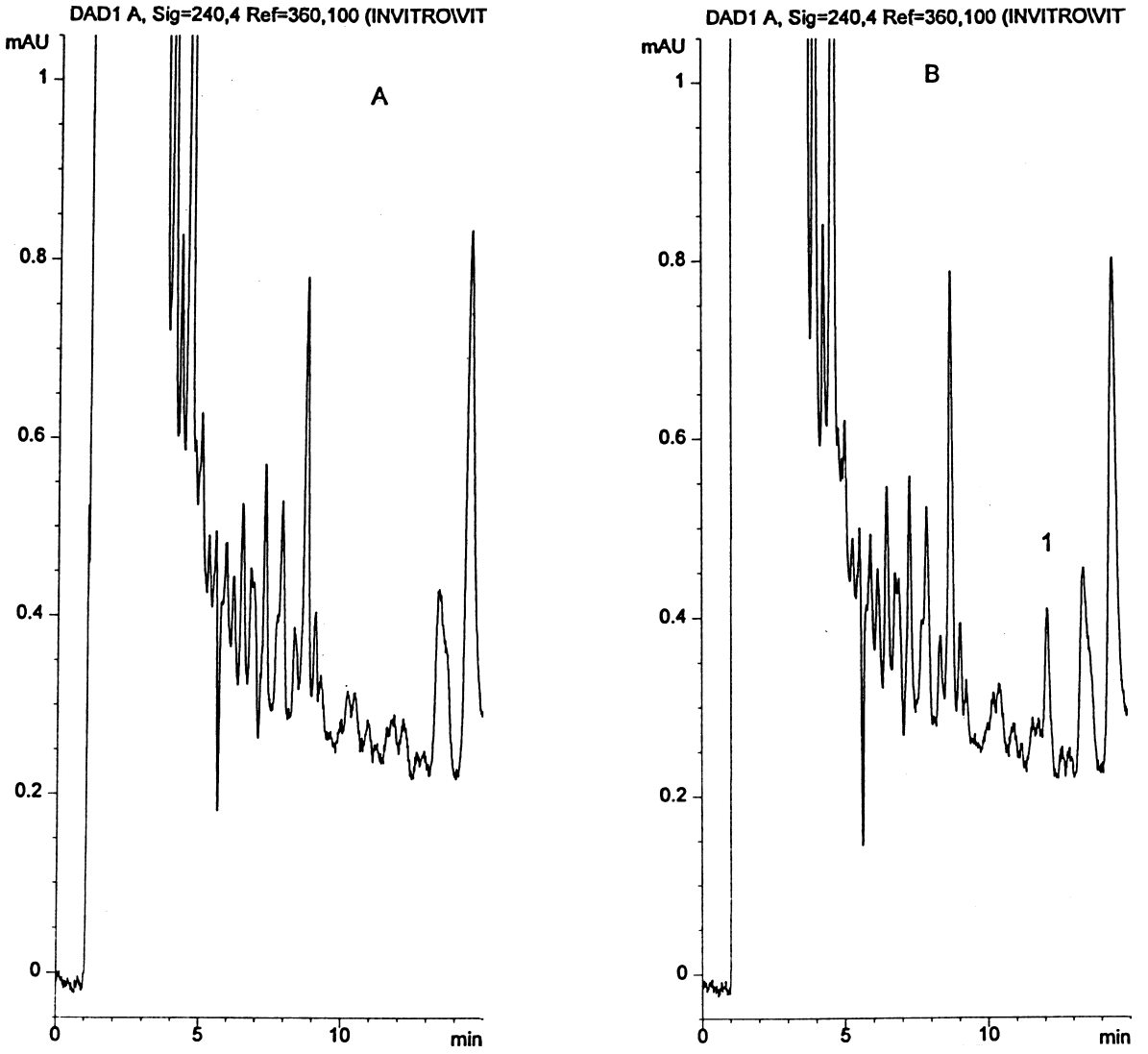

Fig. 4. Limit of detection. Chromatograms of (A) blank human plasma, (B) human plasma spiked with 3 ng/ml of 7-oxo-DHEA-3β-sulfate. Peak: 1=7-oxo-DHEA-3β-sulfate. Chromatographic conditions as mentioned in Section 2.3, mobile phase: System A.

DHEA (II) was found to metabolize very quickly undergoing a phase two conjugation reaction to yield 7-oxo-DHEA-3β-sulfate (IV). Therefore it is safe to conclude that when administered to humans, the acetyl ester of 7-oxo-DHEA is rapidly absorbed and hydrolyzed to liberate free 7-oxo-DHEA (II) and like DHEA (I), the 7-oxo derivative is rapidly converted to the sulfate ester, in which form it circulates in the blood.

The described method has good reproducibility and accuracy necessary for clinical studies.

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Table 1
Within-run and between-run accuracy and precision (% CV) in the measurement of 7-oxo-DHEA-3β-sulfate in human plasma

Spiked concentration (ng/ml)	*n*	Measured concentration (ng/ml)	Precision (% CV)	Accuracy (%)
Within-run				
20	5	20.0	5.9	0.0
50	5	48.8	2.6	−2.4
100	5	103.7	0.8	3.7
500	5	530.2	2.0	6.0
Between-run				
20	22	19.9	16.2	−0.5
50	24	47.0	12.9	−6.0
100	23	95.0	10.5	−5.0
500	8	482.7	7.5	−3.5

Table 2
Stability studies on 7-oxo-DHEA-3β-sulfate in human plasma

Concentration (ng/ml)	Study parameters		
	Room temperature mean (% CV) $n=5$	−20° for 4 months mean (% CV) $n=5$	Freeze–thaw cycles mean (% CV) $n=5$
20	20.4 (3.8)	20.8 (3.5)	17.5 (9.4)
100	102.3 (2.7)	107.6 (2.5)	103.4 (1.0)
500	502.4 (1.9)	562.9 (4.2)	466.0 (9.6)

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