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AN ASSAY OF 4-HYDROXY-*trans*-2-NONENAL IN HUMAN SEMINAL PLASMA USING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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A method is described for the determination of 4-hydroxy-trans-2-nonenal in human seminal plasma. Semen samples were obtained from male partners of couples presenting for a fertility evaluation. After liquefaction, the samples were centrifuged and the seminal plasma was stored at –80 °C. 4-Hydroxy-trans-2-nonenal was derivatized with 1,3-cyclohexanedione to generate the fluorescent alkyl acridine derivative. After derivatization, seminal plasma proteins were precipitated with cold perchloric acid, and the supernatant was injected into the HPLC system. The separation was realized on an analytical reversed-phase column with fluorescence detection. The mixture of ethanol and deionized water was used as the mobile phase. The intra-assay coefficients of variation were below 10 %. Quantitative recoveries from spiked seminal plasma were between 62.0 and 81.0 %.

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Introduction

Oxidative stress induced by reactive oxygen (ROS) and nitrogen (RNS) species has been proposed as one of the potential causes for infertility in men [1-3]. Spermatozoa membranes are particularly susceptible to free radical-induced damage because of their high content of polyunsaturated fatty acids [4,5]. This process, called lipid peroxidation, is a chain reaction involving initiation, propagation and termination. Lipid peroxidation is initiated by the attack of an unsaturated fatty acid by any radical. Fatty acid radical is stabilized by molecular rearrangement to produce a conjugated diene. It reacts with an oxygen molecule forming fatty acid peroxyl radical. Decomposition of the fatty acid peroxyl radical generates secondary lipid peroxidation products such as hydrocarbon gases and aldehydes. Lipid peroxidation decreases the membrane fluidity and changes the phase properties of the membranes. The results are a decreasing number of spermatozoa and a deterioration of sperm motility [6-11].

4-Hydroxy-*trans*-2-nonenal (HNE) is an α,β-unsaturated hydroxyalkenal which is produced by lipid peroxidation of ω-6 polyunsaturated fatty acids, arachidonic acid and linoleic acid. This aldehyde is highly reactive and may be considered to be a toxic molecule. It reacts with biomolecules as amino acids, proteins and nucleic acid bases. For this reason, it is very cytotoxic, genotoxic, carcinogenic and mutagenic [12-14].

Recently, the determination of HNE has attracted increasing interest. Several methods are available for the measurement of HNE in biological samples, but few are suitable for direct analysis in routine use. A commonly used method is high-performance liquid chromatography (HPLC) with various detection techniques, such as spectrophotometric [15,16], fluorimetric [17], electrochemical (EC) [18] and mass spectrometry (MS) [19]. HPLC with ultraviolet and fluorescence detection requires derivatization. Gas chromatographic [20] and micellar electrokinetic chromatographic [21] techniques were described too. Only few methods for determination of HNE in human seminal plasma were developed [22].

The aim of this study was to develop and validate a reliable RP-HPLC method with fluorescence detection for the measurement of HNE in human seminal.

Materials and Methods

Reagents and Chemicals

4-Hydroxy-*trans*-2-nonenal dimethylacetal, 1,3-cyclohexanedione, 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene), perchloric acid, hydrochloric acid, 5-sulfosalicylic acid, metaphosphoric acid and ammonium acetate were

obtained from Sigma (St. Louis, MO, USA). HPLC-gradient grade methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, acetonitrile and *n*-hexane were from Merck KgaA (Darmstadt, Germany). All other chemicals were of analytical grade.

Standard Preparations

500 µl 4-hydroxy-*trans*-2-nonenal dimethylacetal was pipetted into a well-capped 15-ml amber glass tube. Then 9.5 ml *n*-hexane was added and the content was mixed vigorously on a vortex mixer for 1 min. Aliquots (200 µl) were stored at –80 °C.

To 200 µl 4-hydroxy-*trans*-2-nonenal dimethylacetal, 1 ml hydrochloric acid (1 mmol l^{-1}) was added and the solution was vortexed for 1 min. After incubation (5 min, room temperature), 4 ml hydrochloric acid (1 mmol l^{-1}) was added. Work solution of HNE was prepared daily, and its concentration was verified spectrophotometrically using molar absorption concentration of HNE = A/13,750 (mol l^{-1}) at 223 nm.

Instrumentation

Chromatographic analyses were performed with a liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an LC-20AD solvent delivery system, a SIL-20AC$_{HT}$ cooled autosampler, a CTO-20AC column oven, an RF-20A$_{XS}$ fluorescence detector and a CBM-20A system controller. The data were collected digitally using LCsolution chromatography software (Shimadzu, Kyoto, Japan). Spectrophotometric analyses were carried out on a Shimadzu (Kyoto, Japan) UV-1700 PharmaSpec spectrophotometer.

Subject and Samples

Semen samples were obtained from male partners of couples presenting for a fertility evaluation at the Sanus, In Vitro Fertilization Clinic, Pardubice, the Czech Republic. The diagnosis of infertility for these patients was formulated according to the World Health Organization (WHO) guidelines (2010). None of the participants had a serious or chronic disease and took any medications and vitamins on the day of semen collection. All study participants gave written informed consent to participate in this study, which was approved according to the Helsinki Declaration by the Institutional Review Board (Sanus, In Vitro Fertilization Clinic, Pardubice).

Semen Samples Collection

Semen samples were collected into sterile plastic containers by masturbation after a period of sexual abstinence of 2 to 3 days. After 30 min of liquefaction at room temperature, the raw semen specimens were divided into two portions, the first to estimate the sperm concentration, sperm morphology and sperm motility, the second to be centrifuged (900×g, 10 min, room temperature). Seminal plasma was pipetted into a well-capped 1.5-ml amber polypropylene tube containing butylated hydroxytoluene (1 g l^{-1}) and immediately stored at –80 °C.

Sample Preparation

200 µl seminal plasma was pipetted into a well-capped 2.0-mL amber glass tube. 200 µl derivatization agent (0.1 g 1,3-cyclohexanedione and 4.0 g ammonium acetate in 6.0 ml deionized water) was added, the content was mixed vigorously on a vortex mixer for 60 s and incubated at 60 °C for 60 min. After cooling to room temperature, 100 µl reaction mixture was pipetted into a well-capped 1.5-ml polypropylene tube. 900 µl cold protein precipitant (perchloric acid) was added, the solution was vortexed for 60 s and incubated at 4 °C for 10 min. After centrifugation (28,000×g, 4 °C, 10 min), the supernatant was filtered through a nylon filter (pore size 0.20 µm, 4 mm diameter, Supelco, Bellefonte, PA, USA) and transferred into 1.0-ml amber vial.

Chromatographic Analysis

The chromatography of HNE was accomplished using a gradient elution (Fig. 1) on a Discovery HS C_{18}, 150×2.1 mm I.D., 5 µm analytical column fitted with a pre-column filter with pore size of 2.0 µm (Supelco, Bellefonte, PA, USA). All separations were performed at the temperature of 30 °C. Mobile phase A was a mixture of ethanol and deionized water (10:90, v/v). Mobile phase B was 100 % ethanol. Prior the use, both phases were vacuum filtered and degassed using ultrasound. The flow rate was kept constant at 0.160 ml min^{-1}. The optimum response of measured HNE (alkyl acridine derivative) was observed when the excitation and emission wavelengths were 385 nm and 445 nm, respectively. The amount of HNE in standard and seminal plasma samples was quantified from corresponding peak area using LCsolution chromatography software. The concentration of HNE in the seminal plasma samples was determined from the calibration curve.

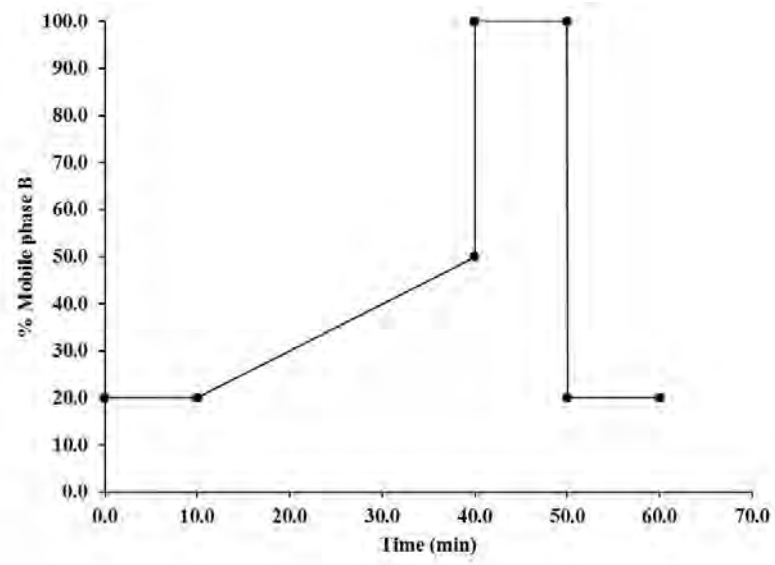


Fig. 1 Gradient of mobile phase: mobile phase B percent vs. time (offset 2.0 min to allow for gradient front reaching the mixer of the column). Mobile phase A, a mixture of ethanol and deionized water (10:90, v/v); mobile phase B, 100 % ethanol

Statistical Analysis

Regression analysis was carried out using the least squares method (software QCexpert, Trilobyte, Pardubice, the Czech Republic).

Results and Discussion

An HPLC with fluorescence detection for the quantification of HNE in human seminal plasma has been presented. Lipid peroxidation may be associated with reduced sperm fertilizing potentials, impaired metabolism, morphology, and motility. HNE is a major aldehydic product of lipid peroxidation, the oxidative degradation of unsaturated fatty acids.

Sample Preparation

The sample preparation is essential for an accurate analysis. Several protein precipitants were tested. Protein precipitant (acetonitrile, ethanol, methanol, propan-1-ol, propan-2-ol, metaphosphoric acid, 5-sulfosalicylic acid and perchloric acid) was carefully added to seminal plasma. After incubation (10 min, 4 °C) and centrifugation (28,000×g, 10 min, 4 °C), the derivatization agent was added to supernatant. Some authors [23-25] used organic solvents as protein precipitants;

however, commercial organic solvents contain contaminants, especially carbonyl compounds. Therefore, a purification step prior to the derivatiza-tion or after is necessary. The effectiveness of solid-phase extraction (SPE) and liquid-liquid extraction (LLE) were tested. LLE is rapid and simple method, HNE derivative in samples extracted with organic solvents (butan-1-ol, *n*-pentane and *n*-hexane) was with difficulties separated from interfering compounds. SPE of the samples was studied using a Discovery DSC-18 sorbent (Sigma, St. Louis, MO, USA). The volume, flow rate, and composition of solutions were studied with the aim of removing the interferences with minimum loss of HNE derivative. Unfortunately, HNE derivative in both standard solution and seminal plasma samples extracted using SPE was with difficulties separated from interfering compounds too. Another alternative is a protein precipitation with acids after derivatization procedure. Only perchloric acid as a protein precipitant led to satisfactory recoveries.

The derivatization procedure was studied in order to achieve the maximum alkyl acridine derivative signal with the lowest number of interferences from the derivatizing reagent CHO. Various temperatures (from 60 °C to 100 °C) and various concentrations of CHO solution (from 0.05 % to 1.00 %) were tested. The optimum results of the derivatization method were obtained using a CHO solution with the concentration of 1.00 % at 60 °C (Figs 2 and 3).

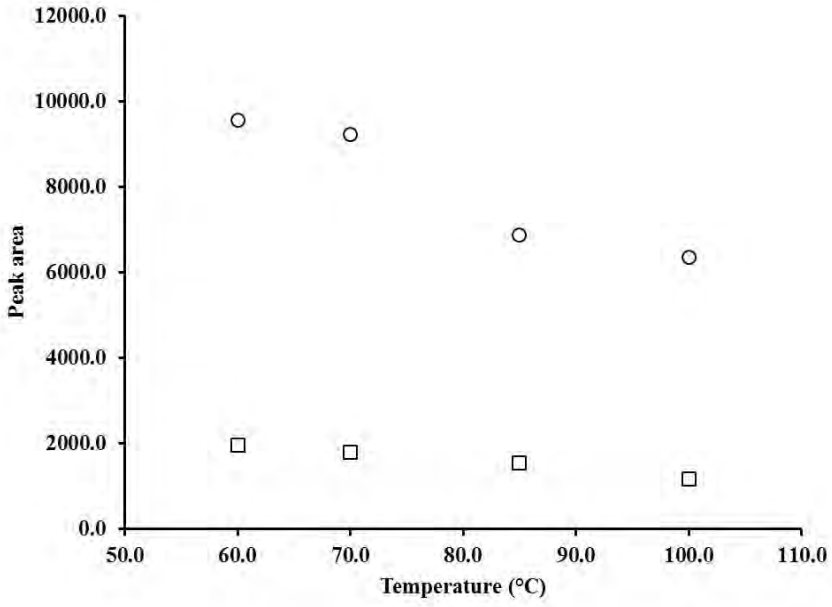


Fig. 2 Optimization of derivatization procedure. Effect of temperature on derivatization recovery; HNE standard solution (○ 5 μmol l^{-1}, □ 1 μmol l^{-1})

HNE is a highly reactive molecule. Hence 4-hydroxy-*trans*-2-nonenal dimethylacetal is shipped. HNE is prepared by acidic hydrolysis of 4-hydroxy-*trans*-2-nonenal dimethylacetal at room temperature. Figure 4 shows the hydrolysis rate of 4-hydroxy-*trans*-2-nonenal dimethylacetal to HNE.

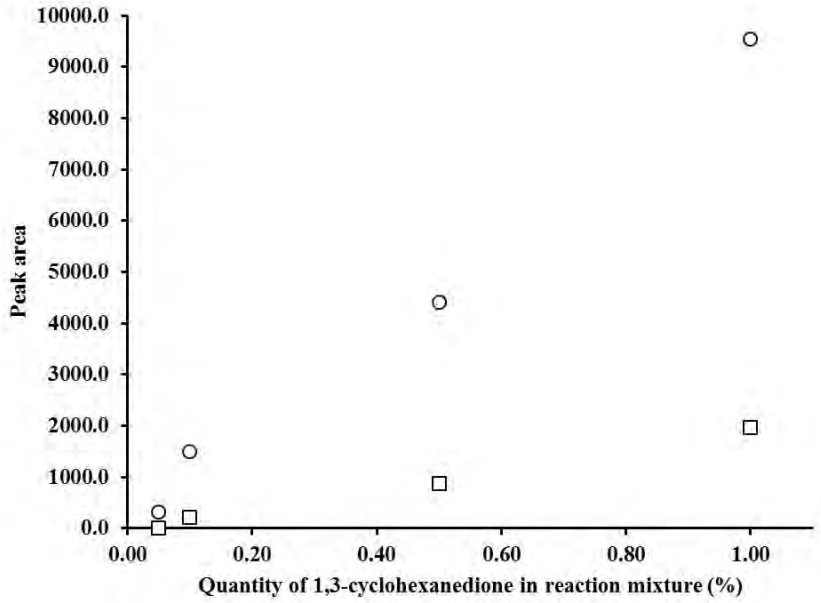


Fig. 3 Optimization of derivatization procedure. The effect of 1,3-cyclohexanedione concentration on derivatization recovery; HNE standard solution (○ 5 μmol l^{-1}, □ 1 μmol l^{-1})

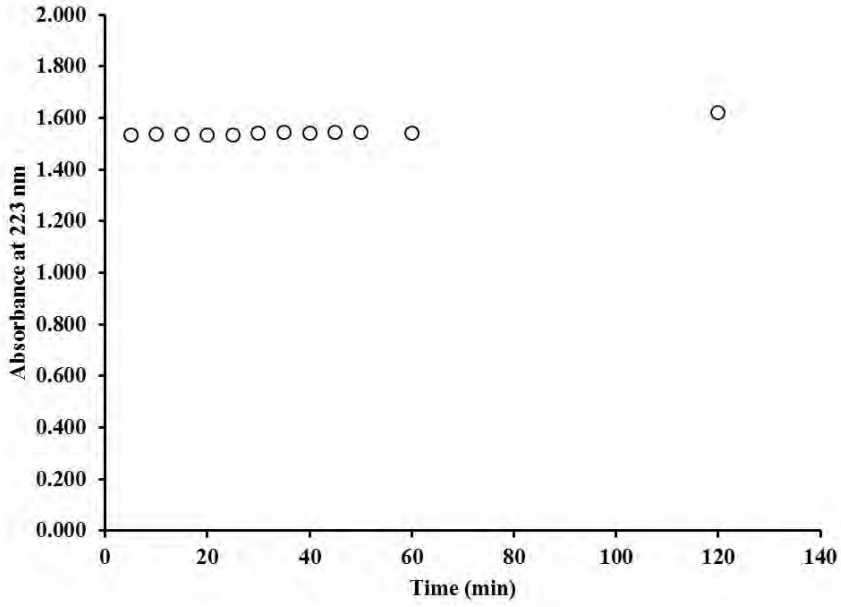


Fig. 4 The effect of 4-hydroxy-*trans*-nonenal dimethylacetal hydrolysis time on HNE recovery

High-Performance Liquid Chromatographic Assay of 4-Hydroxy-*trans*-2-nonenal

HNE after its derivatization with 1,3-cyclohexanedione was separated as the fluorescent alkyl acridine derivative on a reversed-phase column using a gradient

system of ethanol and deionized water. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. A standard solution of HNE as well as pooled seminal plasma samples were used for studying the mobile phase composition. Several eluents (mixtures of organic solvents such as acetonitrile, ethanol, methanol, propan-1-ol and propan-2-ol with deionized water) and several gradients were tested. The best results were obtained for the conditions described in the section "Chromatographic Analysis". Column temperature was changed from 25 to 45 °C. The optimum temperature interval was from 25 to 30 °C. The criteria involved resolution, stability of the fluorescence and analysis duration. According to our results, we can conclude that the presented method is highly robust. Two different columns, both of C18 type, were assayed in this research: a Discovery and a LiChroCART, Purospher STAR (Merck, Darmstadt, Germany). The Discovery column was selected for the further experiments. Two lengths (150 and 250 mm) and two internal diameters (2.1 and 4.0 mm) were assayed, and that of 150 mm length with 2.1 mm internal diameter yielded the best resolution with the shortest retention time for HNE in human seminal plasma. The 150 mm column provided a proper separation between HNE derivative and interferences. The HPLC chromatograms of HNE in standard solution and human seminal plasma are shown in Figs 5 and 6.

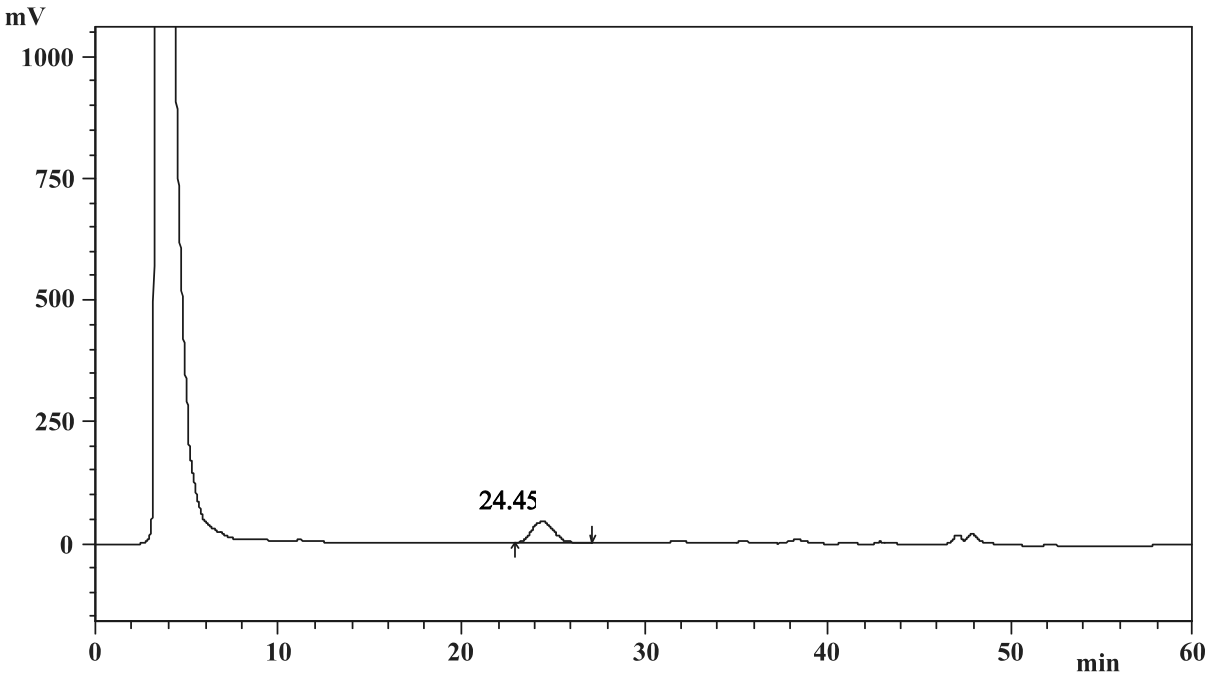


Fig. 5 HPLC chromatogram of HNE (2.10 μmol l^{-1}) in standard solution. HPLC conditions: gradient elution (mobile phase A, 10 % ethanol in deionized water; mobile phase B, 100 % ethanol), stationary phase was analytical column Discovery HS C_{18}, 150×2.1 mm I.D., 5 μm fitted with a pre-column filter (pore size: 2.0 μm), the flow rate was kept constant at 0.160 mL min^{-1}, separation ran at 30 °C and HNE (alkyl acridine derivative) was monitored at excitation and emission lengths of 385 nm and 445 nm, respectively

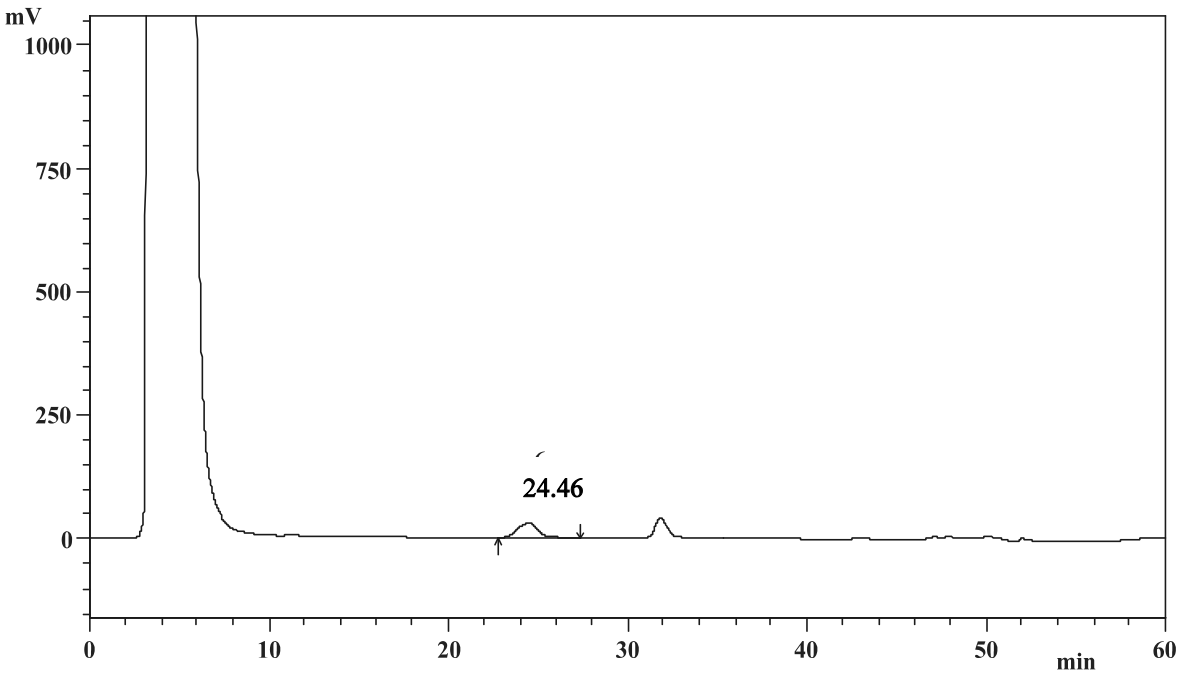


Fig. 6 HPLC chromatogram of HNE (1.45 μmol l^{-1}) in human seminal plasma. For HPLC conditions, see Fig. 5

The precision of HNE analysis in seminal plasma samples is shown in Table I. To determine the same-day precision, the seminal plasma samples were analyzed ten times in the same day under the same conditions. The coefficients of variation were below 10 %. The spike recoveries ranged between 62.0 % and 81.0 % (Fig. 7). The calibration curves (10-point for determining analytical parameters, 0.20-20.00 μmol l^{-1} and 7-point for routine analysis, 0.20-10.00 μmol l^{-1}) were linear over the whole tested range (Fig. 8). The regression line obtained from the combination of five standard curves was $y = 174638x + 8932$. The mean slope, intercept and correlation coefficient (R) for the calibration curve were 174638 (95 % confidence interval, 178952-181935), –0.05 μmol l^{-1} (–0.07 to –0.02 μmol l^{-1}) and 0.9991, respectively. The lowest concentration that could be quantified with an acceptable accuracy and precision was 0.20 μmol l^{-1} (100 fmol $inject^{-1}$). Furthermore, the limit of detection for HNE, defined as a signal-to-noise (S/N) ratio of 3:1, was 0.06 μmol l^{-1} (30 fmol $inject^{-1}$).

Table I Precision of HNE in human seminal plasma

	Mean ± *S.D.*, μmol l^{-1}	*CV*, %
Precision (within-day)		
$n = 10$	1.17 ± 0.10	8.5
$n = 10$	0.89 ± 0.08	9.0

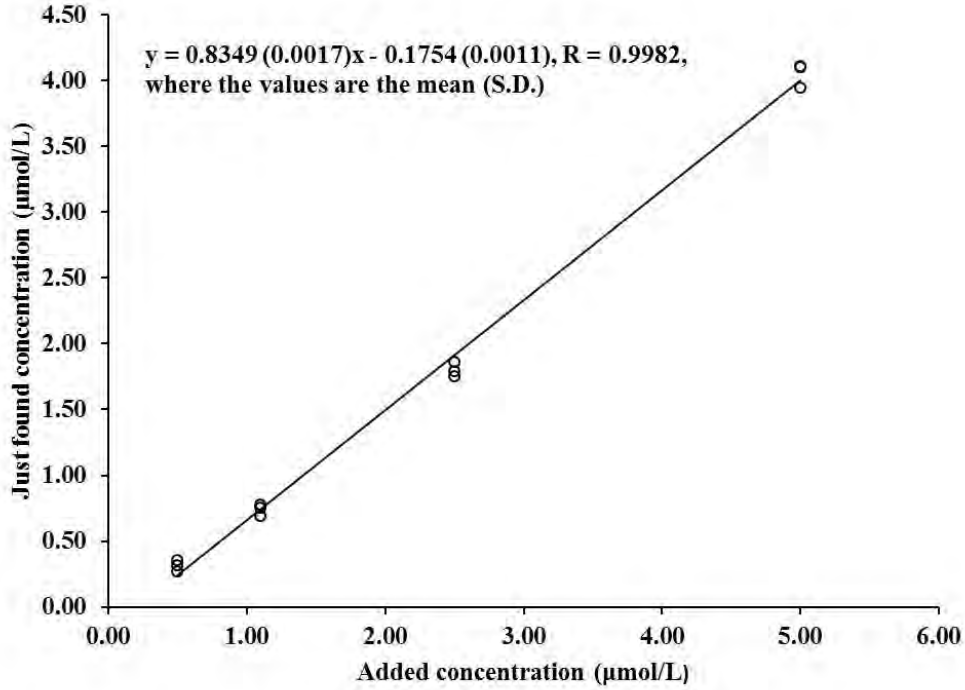
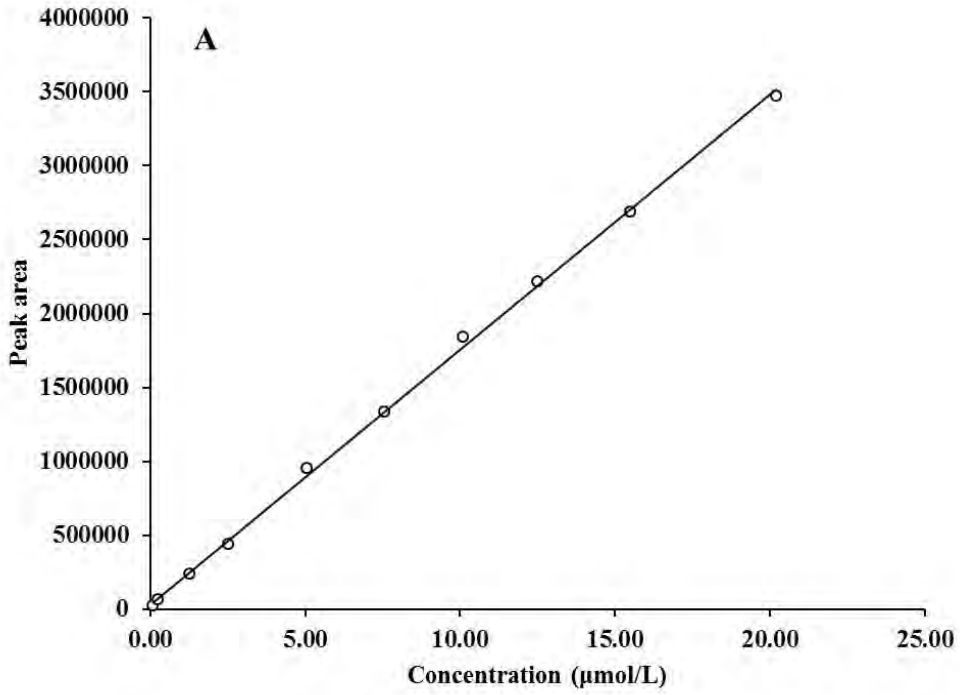


Fig. 7 Recovery experiment. Values of triplicate assays are recorded. Slope corresponds to the mean recovery 70.5 % (*S.D.* = 8.1 %)



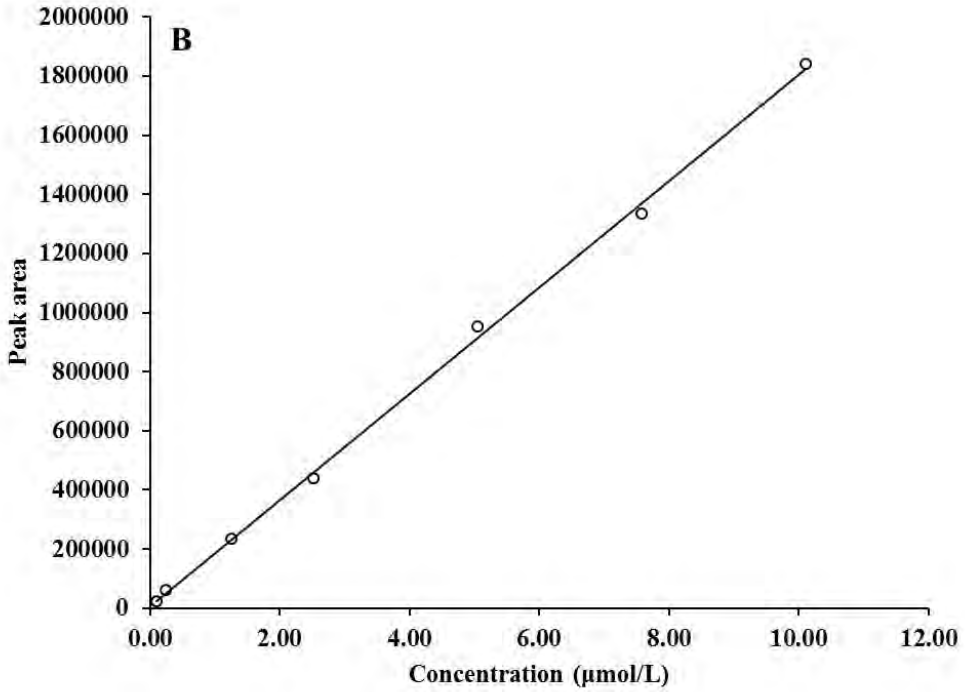


Fig. 8 Typical standard curves for HPLC quantification of HNE. (A) 10-point for determining analytical parameters; (B) 7-point for routine analysis. Regression equations: (A) $y = 172587x + 29569$, $R = 0.9994$; (B) $y = 179755x + 6392$, $R = 0.9993$

Conclusion

An HPLC method with fluorescence detection for the determination of HNE in human seminal plasma was developed. High reactivity of HNE is a major problem in the measurement. The analytical performance of presented method was compared with those previously reported. The most feasible option would be to measure HNE directly. The direct determination of HNE avoids the typical problems associated with the derivatization procedures. Here, HPLC/MS is the method of choice.

Acknowledgment

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