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Note

Selective on-line trace enrichment for the determination of ethynyl steroids in urine by liquid chromatography with precolumn technology

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Ethynyl steroids are being prescribed extensively as contraceptives, to control menstrual disorders and to suppress menopausal symptoms. Because of the toxicity of estrogens, the daily intake via contraceptive applications is reduced to the absolute minimum and, for ethynylestradiol, typically is of the order of 10–50 μg [1]. Ethynylestradiol is partly excreted unchanged in urine, but this occurs very slowly. Consequently, concentration levels in urine are in the low ng/ml range and require very sensitive and selective methods for determination.

Heikkinen et al. [2] described the use of ion-exchange chromatography in steroid analysis prior to gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS). Phenolic steroids were selectively isolated by anion exchangers, and ethynyl steroids by cation exchangers in the silver (I) [Ag(I)] form. Tetsuo et al. [3] reported the analysis of ethynyl steroids, using hydrophobic and ion-exchange [in Ag(I) form] precolumns, followed by derivatization and GC–MS. Recoveries for urine spiked with non-conjugated steroids were 80–90%, and the detection limit was ca. 5 pg/ml. Andersson et al. [4] described an improved method for the clean-up of ethynylestradiol from urine. The glucuronides were enzymatically hydrolysed, and the released hormones were trapped on a hydrophobic sorbent. Next the solutes were eluted and selectively reconcentrated on a cation-exchange sorbent in its Ag(I) form. Thanks to the use of a Ag(I)-loaded precolumn the ethynyl steroids could be selectively isolated, even in the presence of relatively high concentrations of endogeneous steroids. The

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main disadvantage of the quoted methods is the use of many off-line sample manipulations, which results in a rather long analysis time. In addition, derivatization is necessary, and expensive GC–MS systems are used.

Recently, we [5] used a small precolumn packed with a silver(I)-8-hydroxyquinoline [Ag(I)-oxine] modified hydroxyallylmethacrylate gel for the selective on-line trace enrichment of a herbicide from river water samples. Buturon, the only phenylurea herbicide that has an ethynyl group, is able to interact selectively with the Ag(I)-loaded precolumn. The application of a Ag(I)-loaded precolumn to urine samples is, however, more problematic because of the high concentration of interfering compounds, e.g. chloride ions and amino acids. By using a dual-precolumn approach, these interferences may be eliminated in an automated way, as was recently demonstrated in the clean-up of barbiturates from urine [6], viz. via a solid-phase extraction procedure involving a hydrophobic sorbent prior to the selective, i.e. Ag(I)-oxine, precolumn.

Liquid chromatographic (LC) methods for the determination of ethynyl steroids in urine are often hindered by the poor resolution between norethindrone, norgestrel and ethynylestradiol. Papas et al. [7] used a ternary mobile phase to obtain sufficient resolution between norethindrone and ethynylestradiol. However, by monitoring at two wavelengths simultaneously, ethynylestradiol can be selectively determined because its absorbance maximum occurs at a wavelength different from that for norgestrel and norethindrone, i.e. 280 nm versus 240 nm.

Obviously, there is still a need for a rapid and automated clean-up procedure for the determination of ethynyl steroids in urine at trace levels. We developed such a method by combining selective on-line trace enrichment using hydrophobic and Ag(I)-loaded sorbents, with LC and dual-wavelength detection.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps, both equipped with home-made membrane pulse dampeners, an MCS 670 Tracer valve switching unit and a Model 200 programmer were used, in combination with two Kratos (Ramsey, NJ, U.S.A.) Model Spectroflow 757 UV absorbance detectors. Chromatograms were either analog recorded at 240 and 280 nm using two Kipp & Zonen (Delft, The Netherlands) BD 40 recorders, or digital via an Anacomp (Kontron) Model 220 computer.

Stationary phases and columns

On-line trace enrichment and clean-up was performed on Chrompack (Middelburg, The Netherlands) 10×2.0 mm I.D. preconcentration columns packed by hand using a syringe containing a slurry of 10 μm styrene–divinylbenzene copolymer PRP_1 (Hamilton, Reno, NV, U.S.A.) or 25–40 μm Ag(I)-loaded Spheron Oxine 1000 (Lachema, Brno, Czechoslovakia) in methanol.

A 100×3 mm I.D. glass cartridge prepacked with 5-μm Chromspher (Chrompack) octadecyl-bonded silica and equipped with a 10×2 mm I.D. guard column packed with 5-μm LiChrosorb RP-18, served as analytical column.

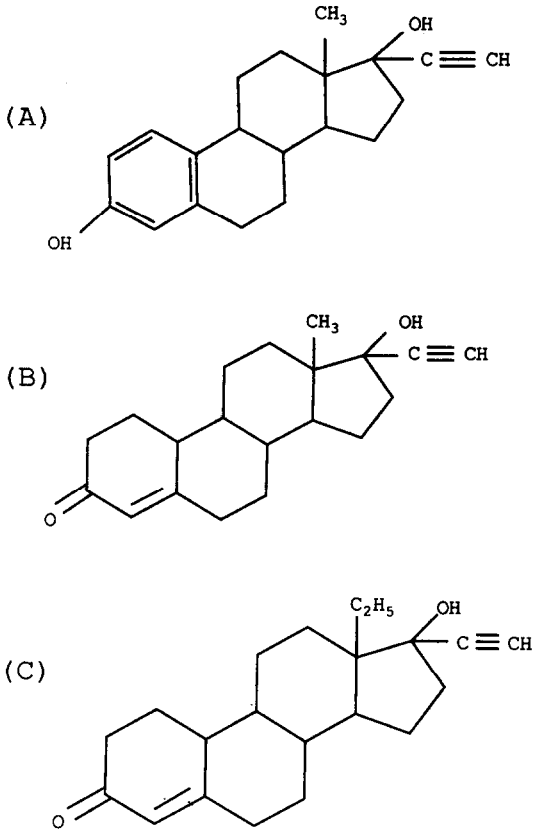

Fig. 1. Structures of ethynyl steroids under investigation: (A) ethynylestradiol; (B) norethindrone; (C) norgestrel.

Preparation of the Ag(I)-oxine phase

An appropriate amount of silver(I) acetate was dissolved in 100 ml of 10^{-3} *M* acetic acid in order to obtain a 0.05 *M* solution. The pH value thus obtained should be ca. 6.2. Then 0.5 g of Spheron Oxine 1000 were added and the mixture was mechanically shaken for 2 h. The Ag(I)-loaded phase was collected on a glass filter and washed with 30 ml of LC-grade water followed by 30 ml of methanol, and dried at room temperature in the dark. The Spheron Oxine material could be regenerated by washing with acetonitrile–water (50:50), acidified with perchloric acid (pH 1.9) followed by water and methanol. After it has been dried at room temperature, the oxine phase is ready for re-loading with Ag(I).

Chemicals

Analytical-grade acetic acid, nitric acid, perchloric acid and ethanol, and HPLC-grade methanol and HPLC-gradient-grade acetonitrile were obtainend from J.T. Baker (Deventer, The Netherlands). Silver acetate was obtained from Aldrich (Beerse, Belgium). Pharmaceutical-grade ethynylestradiol, norgestrel and norethindrone were obtained from Sigma (St. Louis, MO, U.S.A.); their structures are given in Fig. 1. A stock solution of the steroids ethynylestradiol (2.5

μg/ml), norgestrel (5 μg/ml) and norethindrone (5 μg/ml) was prepared in ethanol, stored at 4°C in the dark and diluted with LC-grade water or urine, prior to use. Urine samples were paper-filtered prior to the on-line trace enrichment.

LC-grade water was prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system. Eluents were degassed under vacuum in an ultrasonic bath. The pH was adjusted after the addition of acetonitrile using a Philips (Eindhoven, The Netherlands) PW 9409 pH meter.

General procedure

Final experiments were performed using the set-up shown in Fig. 2. The procedure is as follows. The PRP_1 precolumn is wetted by 5 ml of methanol and conditioned with 10 ml of water via pump A at 5 ml/min. Then 20 ml of a standard steroid mixture or a urine sample are loaded onto the PRP_1 precolumn at 4 ml/min. Here sorption of the ethynyl steroids and of various organic contaminants occurs, while inorganic and water-soluble constituents, e.g. chloride ions and amino acids, are flushed to waste. The PRP_1 precolumn is flushed with 10 ml of water and 10 ml of methanol–water (50:50) in order to ensure that contaminants that might interfere with the sorption process on the second precolumn are flushed to waste. Next, the Ag(I)-oxine precolumn is conditioned with 2 ml of methanol–water (70:30). The steroids are eluted with another 4 ml (in the forward-flush mode, at 1.0 ml/min) from the PRP_1 precolumn to the Ag(I)-oxine precolumn on which they are selectively trapped in a narrow zone by complexation, while organic interferences, e.g. endogeneous steroids not having the ethynyl group, are flushed to waste. Then the Ag(I)-oxine precolumn is flushed with 2 ml of methanol–water (70:30) followed by 2 ml of water at 2 ml/min [the latter in order to remove all methanol prior to the next (elution) step]. Finally, the ethynyl steroids are desorbed in the backflush mode by 2.4 ml of acidified (pH 1.9) acetonitrile–water (15:85) via pump B and transferred to the C_{18} analytical column (which has been conditioned with the same mobile phase), on top of which peak compression occurs. The separation is achieved by a step gradient to acetonitrile–water (50:50), and the hormones are detected by UV absorbance at 240 and 280 nm. In the present study, the Ag(I)-oxine precolumns were replaced by hand prior to the next run. However, complete automation can easily be obtained by using a microprocessor-controlled cartridge exchange device, as recently described by Nielen et al. [9].

In order to increase sample throughput, the next run can be started while the actual separation is still in progress. Consequently, an analysis takes only 30 min. The general procedure is summarized in Table I; details of the valve-switching program will be made available on request.

RESULTS AND DISCUSSION

Characteristics of the precolumns

Breakthrough experiments were carried out according to the procedure described in ref. 8, using a 250 ng/ml standard solution of ethynylestradiol in water. At a flow-rate of 5 ml/min the breakthrough volume on the 10×2 mm I.D.

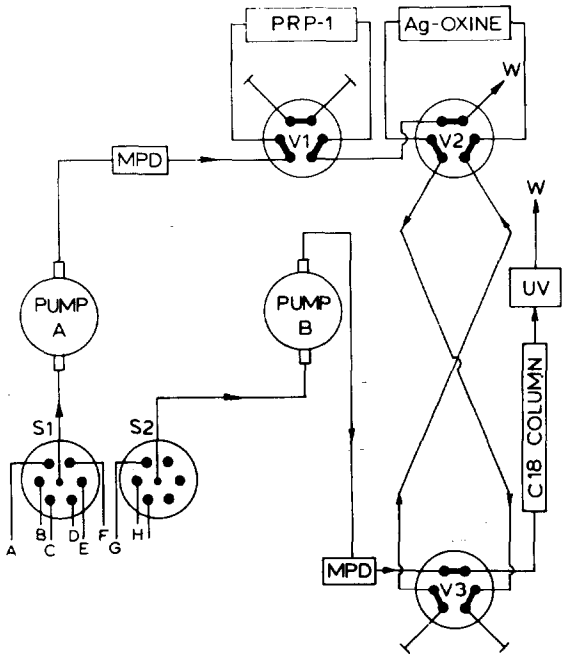

Fig. 2. Experimental set-up for the on-line trace enrichment and determination of ethynyl steroids in urine. S_1 and S_2 = low-pressure solvent selection valves; V_1, V_2 and V_3 = high-pressure switching valves; MPD = membrane pulse dampener; W = waste; A = water; B = methanol; C = water; D = sample; E = methanol–water (50:50); F = methanol–water (70:30); G = acetonitrile–water (15:85) acidified with perchloric acid (pH 1.9); H = acetonitrile–water (50:50). Precolumns: 10×2.0 mm I.D. PRP_1 and Ag(I)-oxine. Analytical column: 100×3.0 mm I.D. C_{18}, equipped with a 10×2.0 mm I.D. guard column. Flow-rate of pump A, 0.5–5 ml/min; flow-rate of pump B, 0.6 ml/min.

PRP_1 precolumn was found to be more than 50 ml, which was the maximum volume tested.

The choice of the eluent for the desorption of the steroids from the PRP_1 precolumn and their subsequent sorption on the Ag(I)-oxine precolumn was taken from ref. 4. Methanol–water (70:30) provided a satisfactory desorption from the PRP_1 precolumn; in addition, the breakthrough volume for ethynylestradiol dissolved in this eluent on the 10×2 mm I.D. Ag(I)-oxine precolumn was found to be more than sufficient: at least 20 ml at a flow-rate of 0.5 ml/min. It should be noticed that retention on the Ag(I)-oxine is dependent on the flow-rate [5]; for safety reasons we used always 1.0 ml/min during this step of the solid-phase extraction procedure.

The mobile phase used for the desorption of the ethynyl steroids from the Ag(I)-oxine precolumn was found to be more problematic. We used a relatively low acetonitrile content in order to be sure to obtain a peak compression effect (cf. below) at the top of the C_{18} analytical column. The peak shape improved considerably after a step gradient from 15 to 50% acetonitrile, which had been substituted for an isocratic elution at a 40% modifier content. The pH of the mobile phase used for desorption from the Ag(I)-oxine was very critical. At a pH of over

TABLE I

GENERAL PROCEDURE USING THE SET-UP OF FIG. 2

1. Trapping of the ethynyl steroids on PRP_1.
2. Flushing of PRP_1 with water and methanol–water (50:50).
3. Transfer of steroids to Ag(I)-oxine with methanol–water (70:30).
4. Flushing of Ag(I)-oxine with methanol–water (70:30) and with water.
5. Conditioning of C_{18} analytical column with acidified acetonitrile–water (15:85).
6. Backflush desorption from Ag(I)-oxine to the C_{18} analytical column using acidified acetonitrile–water (15:85).
7. Step-gradient to acetonitrile–water (50:50).

2.5, no desorption occurred at all. We used acetonitrile–water (15:85) acidified to pH 1.9, but still at least 2 ml of eluent were necessary in order to obtain satisfactory desorption. Probably the unfavourable elution profile is not due to the pH value but to the low acetonitrile content, which is not able to provide an efficient elution because of the relatively strong reversed-phase nature of the bare Spheron Oxine. In practice, however, this drawback could be easily overcome by using backflush desorption and creating peak compression at the top of the analytical column.

The choice of the acid for desorption was not critical; both nitric and perchloric acid yielded acceptable results. This indicates that the desorption from the Ag(I)-oxine is primarily a neutralization process. We preferred the use of perchloric acid because of its lower background at low detection wavelengths.

Performance and application

With the experimental set-up shown in Fig. 2 and the general procedure described in Table I, analytical data were collected for ethynylestradiol. norgestrel and norethindrone. The results, which are presented in Table II, clearly demonstrate the potential of the method.

TABLE II

GENERAL PERFORMANCE OF THE AUTOMATED ANALYSIS OF ETHYNYL STEROIDS ACCORDING TO THE PROCEDURE IN TABLE I AND USING THE SET-UP OF FIG. 2

Samples (20 ml) were spiked at the 5–10 ng/ml level.

Compound	Repeatability ($n=3$) (% R.S.D.)		Recovery ($n=3$) (%)		Detection limit* ($n=3$) (ng/ml of urine)
	Standard	Urine	Standard vs. loop (peak area)	Urine vs. standard (peak height)	
Norethindrone	2.5	2.2	72	86	0.10
Ethynylestradiol	2.7	3.5	67	92	0.25
Norgestrel	3.4	3.4	76	102	0.12

*Signal-to-noise ratio = 3:1.

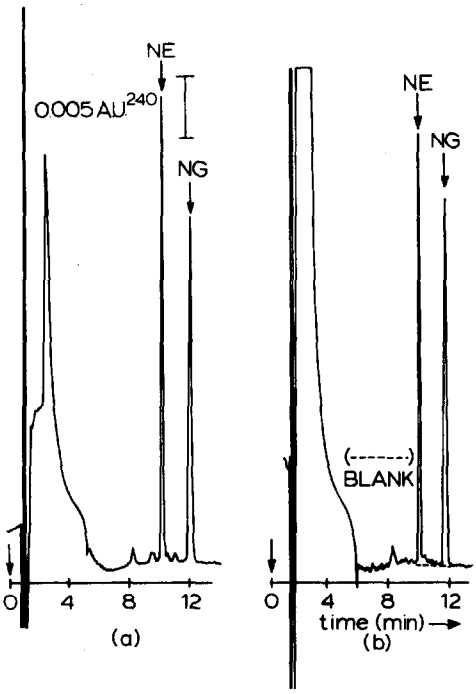

Fig. 3. Chromatograms of 20-ml samples spiked with 10 ng/ml norethindrone (NE), norgestrel (NG) and 5 ng/ml ethynylestradiol (EE), and analysed according to the procedure in Table I using the set-up of Fig. 2. Detection at 240 nm. Samples: (a) standard; (b) urine.

The recovery for the complete sorption–desorption procedure was determined by comparing the peak areas obtained with a direct 113-μl loop injection onto the C_{18} analytical column and a 20-ml on-line trace enrichment of the same (but proportionally diluted) sample, using the system in Fig. 2 and the procedure in Table I. The recovery for standard solutions was found to be satisfactory but not quantitative. The losses are probably due to incomplete desorption from the Ag(I)-oxine precolumn. In spite of this the repeatability was very good; therefore no internal standard was required.

The recovery from spiked urine compared with that from a standard solution was between 86 and 102%. The repeatability for spiked urine samples was between 2.2 and 3.5%. Detection limits in urine samples are at the sub-ng/ml level.

Figs. 3 and 4 show chromatograms obtained with the UV absorbance detectors set at 240 and 280 nm, respectively. One should note that, in the latter case, the detector sensitivity has to be increased ten-fold because of the lower molar extinction coefficient of ethynylestradiol. Figs. 3a and 4a were obtained from 20-ml standard samples spiked with 10 ng/ml norethindrone and norgestrel, and 5 ng/ml ethynylestradiol. The selectivity obtained by the use of two different wavelengths is demonstrated by the absence of ethynylestradiol at 240 nm and the small norethindrone and norgestrel peaks at 280 nm. Figs. 3b and 4b were obtained from 20-ml urine samples spiked at the same level as the standard. The excellent recovery compared with the standard solution is obvious, and the good selectivity

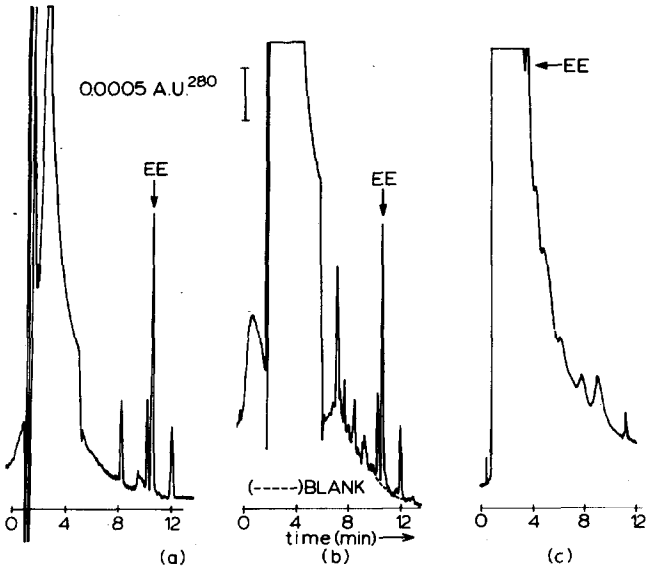

Fig. 4. Analyses of the same samples as in Fig. 3, except for the detection wavelength (280 nm) and a ten-fold higher detector sensitivity. (c) Urine sample but using the PRP_1 precolumn only.

allows the identification of these hormones by their UV characteristics in combination with the retention times.

Fig. 4c shows a chromatogram for the same urine sample as analysed before but with the use of the PRP_1 precolumn only. The precolumn was flushed with 10 ml of water and methanol–water (50:50) before the steroids were desorbed isocratically by acetonitrile–water (40:60) to the C_{18} analytical column and detected at 280 nm. The benefit of selective trace enrichment using the dual-precolumn approach is clearly evident for the determination of ethynylestradiol in real samples.

CONCLUSIONS

When two precolumns are used in series, to remove (i), inorganic and other water-soluble compounds and (ii) organic compounds, urine samples can be analysed for ethynyl steroids with good sensitivity and selectivity, even when UV detection at low wavelengths is used. Additional selectivity is obtained by recording at two different detection wavelengths simultaneously. The present automated method allows the rapid screening of urine samples for ethynyl steroids without time-consuming clean-up and derivatization, and without the use of expensive GC–MS equipment [3,4].

The remaining off-line manipulations, i.e. the replacing of the precolumns and the hydrolysis of conjugates (necessary in case of patient samples), can be also automated by using a microprocessor-controlled cartridge exchanger [9] and a column packed with immobilized glucuronidase [10,11], respectively. The application of immobilized enzymes to the automated de-glucuronidation of large volumes of urine is currently under investigation.

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