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Determination of estramustine phosphate and its metabolites estromustine, estramustine, estrone and estradiol in human plasma by liquid chromatography with fluorescence detection and gas chromatography with nitrogen-phosphorus and mass spectrometric detection

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Abstract

Bioanalytical methods for the determination of estramustine phosphate by liquid chromatography and its four main metabolites estromustine, estramustine, estrone and estradiol by gas chromatography are described. For the estramustine phosphate assay the plasma was purified by protein precipitation followed by a C_{18} solid-phase extraction. For the metabolite assay the plasma samples were purified by a C_{18} solid-phase and liquid–liquid extraction procedure and derivatised by silanization. Thereafter, estramustine and estromustine were quantified by gas chromatography with nitrogen-phosphorus detection and estradiol and estrone were quantified by gas chromatography with selected ion monitoring. The methods were validated with respect to linearity, selectivity, precision, accuracy, limit of quantitation, limit of detection, recovery and stability. The limit of quantitation was 2.3 μmol/l for estramustine phosphate, 30 nmol/l for estromustine and estramustine, 12 nmol/l for estrone and 8 nmol/l for estradiol. The results showed good precision and accuracy for estramustine phosphate and the four metabolites. The intermediate precision was 6.2–13.5% (C.V.) and the accuracy was 91.8–103.9%.

Keywords: Estramustine phosphate; Estromustine; Estrone; Estradiol; Estramustine

1. Introduction

Estramustine phosphate (Estracyt®, Emcyt, EMP) is a nornitrogen mustard derivative of estradiol-17β-phosphate (Fig. 1), which has been in use for several years for the treatment of advanced prostrate cancer [1–5]. The drug is an antimitotic agent with estrogenic properties.

EMP is readily dephosphorylated to estramustine (EaM) in vivo [5–7]. EaM is then oxidized in the 17th position to estromustine (EoM) and the antimitotic effects are exerted by these two metabolites. EoM is the main plasma metabolite in prostrate cancer patients [9–11]. The estrogenic properties of EMP are due to the metabolites estradiol (E2) and estrone (E1) formed by cleavage of the carbamate

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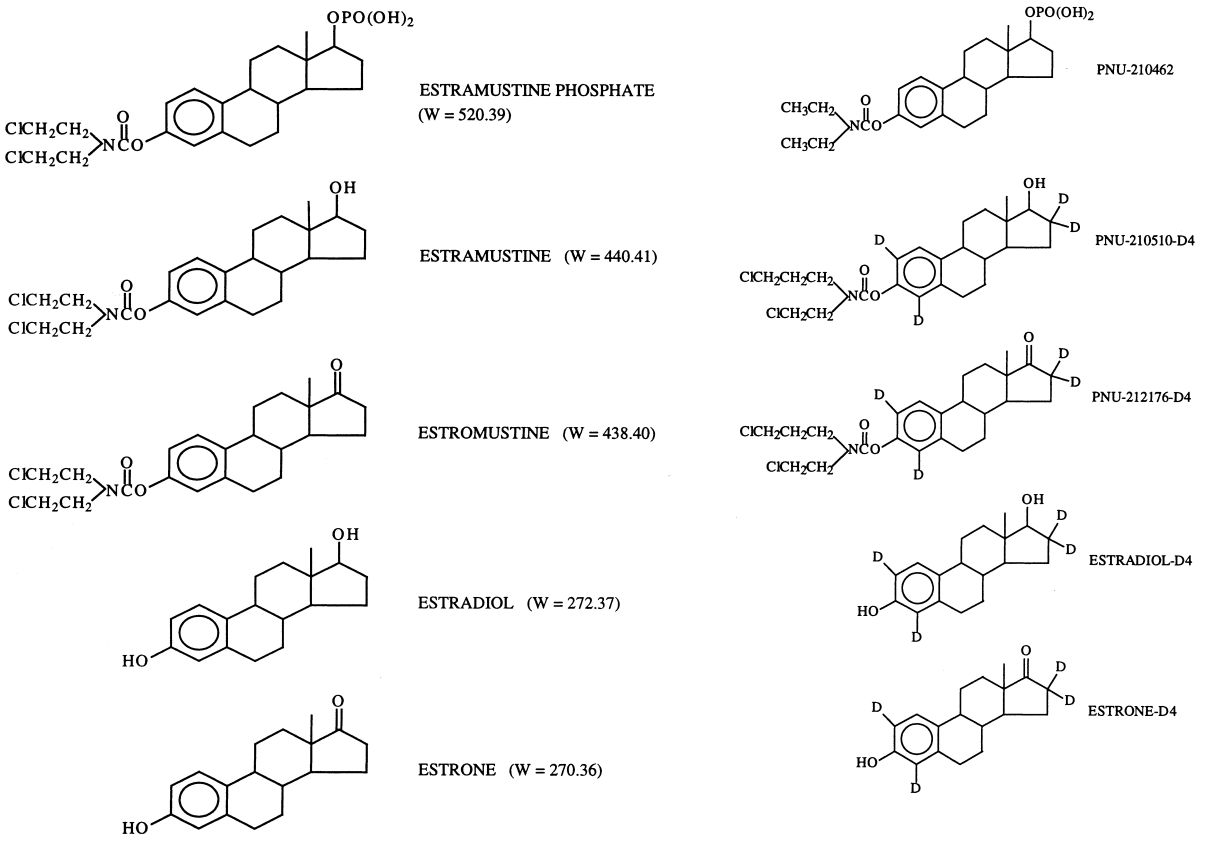


Fig. 1. Estramustine phosphate with metabolites and internal standards.

ester of estramustine and estromustine respectively [5–8,12,13].

Packed column gas chromatographic and liquid chromatographic methods have previously been used for the determination of the EMP metabolites [14,15]. Packed column GC or LC with fluorescence (or UV) detection did, however, not provide enough sensitivity for the determination of low concentrations of the metabolites (possibly with the exception of estradiol, which fluoresces strongly). Furthermore due to a rising number of samples there was also a need to increase the sample throughput. The former EMP assay was a radio immunoassay (RIA) method, but the assay cross determined the estrogens [16].

Therefore, the aim of the present study was primarily to develop a method for EMP as well as a new method for the active metabolites EaM, EoM, E2 and E1 with improved sensitivity as compared to the previous methods. A secondary aim was to increase the sample throughput of the metabolite method.

HPLC with fluorescence detection was chosen for estramustine phosphate, which is usually present in relatively high concentrations but is not volatile. Using GC–MS, estromustine gives a very small molecular peak and only low mass fragments, why GC–NPD was considered as a more sensitive and selective detection technique for the determination of estromustine and estramustine. Unfortunately, the NPD technique was not applicable for the determination of estrogens, why a third technique (MS) had to be chosen for their detection.

2. Experimental

2.1. Materials

EMP, the metabolites EoM, EaM and the internal standards PNU-210462, PNU-212176-D_4, PNU-210510-D_4, E1-D_4 and E2-D_4 (see Fig. 1) were synthesised at the Pharmacia & Upjohn laboratories in Helsingborg. E1 was purchased from Merck, Darmstadt, Germany and E2 from Diosynth BV, Oss, The Netherlands. Methanol, acetonitrile, tetrahydrofuran and diethyl ether were of high-performance liquid chromatographic (HPLC) grade and toluene was of analytical grade (Labscan, Dublin, Ireland), xylene, acetic acid (99.8%), ammonia (25%), were of analytical grade, (BDH, Poole, UK). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA, No 3-3027) was purchased from Supelco Inc. (Bellafonte, PA, USA). Tetrabutyl ammonium hydroxide (TBA, 40 wt%) was purchased from Janssen Chimica, Geel, Belgium. Water was deionized by a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were purchased from standard sources and used without further purification. Bond Elut C_{18} extraction columns (500 mg, 2.8 ml and 10 ml) were purchased from Analytichem International (Harbor City, CA, USA).

2.2. Equipment

The HPLC system used for the EMP assay consisted of a Varian 9020 pump (Varian, Sunnyvale, CA, USA), a Waters 717 WISP autosampler (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA), a Spectroflow 980 fluorescence detector (Applied Biosystems, Ramsey, NJ, USA) and a Waters Millennium 2020 Chromatography Manager.

The HPLC column was Waters Novapak C_{18} radial Pak LC-cartridge, 8 mm I.D., 10 cm, 4 μm.

The pump operated at 1.0 ml/min. A gradient of tetrahydrofuran–phosphate buffer (pH 3.0; 0.1 *M* with 1.4 m*M* TBA) with an increasing content of tetrahydrofuran from initially 10% (v/v) to 55% (v/v) in 25 min and kept at 55% for 5 min was used. The column was maintained at ambient temperature (22–24°C). The fluorescence detector operated at an excitation wavelength of 200 nm and with an emission 325 nm bandpass filter.

The gas chromatographic (GC) system, used for determination of EaM and EoM, consisted of a Hewlett-Packard (Avondale, PA, USA) HP 5890A gas chromatograph with split/splitless injector and nitrogen-phosphorus detector (NPD) equipped with an HP 7673A autosampler and a Waters Millennium 2020 Chromatography Manager.

The GC column was a 15 m Durabond DB-1, 0.32 mm I.D., 0.25 μm layer purchased from J&W Scientific (Folsom, CA, USA). Helium (purity 4.6) was used as the carrier gas and the linear flow was set to 33 cm/s at 280°C. The injector temperature was set to 270°C. The oven temperature was programmed from 135°C to 280°C at a rate of 30°C/min and kept at 280°C for 15 min. The detector temperature was set to 300°C.

The chromatographic system, used for determination of the estrogens, consisted of a Hewlett-Packard HP 5890 gas chromatograph-Finnigan Mat SSQ 710 mass spectrometer (Finnigan MAT, San José, CA, USA) equipped with split/splitless injector and an autosampler (Finnigan Mat A200S).

The GC column was a 25 m HP-5, 0.2 mm I.D., 0.33 μm layer purchased from Hewlett-Packard. The carrier gas used was helium (purity 4.6). The inlet pressure was adjusted to give retention times of 13.0–13.5 min for estrone and 13.5–15.2 min for estradiol. The oven temperature was programmed from 105°C to 290°C in a two step gradient at a rate of 25°C/min to 240°C and 5°C/min to 290°C kept at 290°C for 2 min. Injector temperature was 250°C, The transfer line was kept at 280°C and the ion source at 150°C.

The trimethyl-silyl derivatives of E1 (E1-TMS) and E2 (E2-di-TMS) were detected as molecular ions at $m/z=342$ and $m/z=416$ and the internal standards, containing four deuterium atoms, at mass numbers four units higher.

2.3. Sample preparation

2.3.1. EMP assay

A plasma volume of 1.00 ml was pipetted to a test tube, and 100 μl of internal standard solution (240 μg PNU-210462/ml in 70% methanol (aq)) was

added. After 15 min of equilibration a volume of 0.75 ml of acetonitrile was added to precipitate the proteins, and the sample was mixed for 5 min. The sample was then diluted with 3.25 ml of 0.1 *M* phosphate buffer pH 7.0, vortex mixed and centrifuged for 10 min at 1450 *g*.

The clear liquid phase was transferred to a 10 ml Bond Elut C_{18} cartridge, activated by flushing with 3 ml of methanol followed by 2 ml of 0.1 *M* phosphate buffer, pH 7.0. The solid residue was suspended in another 0.75 ml of acetonitrile+3.25 ml of 0.1 *M* phosphate buffer, mixed and again centrifuged. Also the second aliquot was transferred to the same extraction cartridge.

The sample was passed through under vacuum, and the sorbent was washed with 2 ml of 1% acetic acid followed by 2 ml of 40% methanol (aq). The compounds were eluted with two volumes (2+1 ml) of 0.13% ammonia in 95% methanol.

The eluate was evaporated to dryness under a stream of nitrogen at 65°C and the residue was dissolved in 200 μl of methanol. The sample was transferred to a LC micro vial, and 10 μl was injected onto the HPLC system.

2.3.2. Metabolite assay

A plasma volume of 1.00 ml was pipetted to test tubes containing 0.5 ml of 0.5 *M* phosphate buffer pH 7.5 and 100 μl of a mixture of the internal standards (PNU-212176-D_4 14 μg/ml, PNU-210510-D_4 19 μg/ml, E1-D_4 8 μg/ml and E2-D_4 5 μg/ml in ethanol). A water volume of 1.00 ml was added.

The mixture was vortex-mixed and applied onto a 3 ml Bond Elut C_{18} cartridge, activated by flushing with 3 ml of methanol and 2 ml of 0.1 *M* phosphate buffer pH 7.5. The sample was passed through under vacuum, and the sorbent was washed with 2 ml of 0.1 *M* phosphate buffer pH 7.5 followed by 1 ml of water and 2 ml of 50% methanol (aq). The compounds were eluted with two volumes (2+1 ml) of methanol.

The eluting solvent was evaporated under a stream of nitrogen at 40°C until about 200 μl remained. Volumes of 1 ml of water and 4 ml of diethylether were added to the residue, and the mixture was extracted on laboratory shaker for 4 min. The test tube was centrifuged, and the organic phase was transferred to a conical test tube and evaporated to dryness.

The dry residue was dissolved in 100 μl of pyridine and derivatised with 100 μl of BSTFA for 60 min at 60°C or over night at room temperature. A volume of 50 μl was transferred to GC-vials for GC–MS, and the remainder of the sample was transferred to GC-vials for GC–NPD, whereafter both aliquots were evaporated to dryness.

The residue of the GC–MS-sample was dissolved in 25 μl of toluene, and 2 μl were injected onto the GC–MS instrument for E1 and E2 determination. The residue of the GC–NPD sample was dissolved in 25 μl of xylene, and 1 μl was injected onto the GC equipped with NPD for EoM and EaM determination.

2.4. Calibration

2.4.1. EMP assay

Calibration standards were run together with every batch of samples. The calibration standards were prepared by spiking blank human plasma to nine different concentrations in the range of 0.6–175 μmol/l of EMP. The standards were processed as described above for the samples. Calibration curves of EMP were established by a Millennium chromatography manager interfaced to the HPLC system. The peak-area ratios of EMP to PNU-210462 versus the concentration in the calibration standards were plotted.

Prevalidation results indicated that the EMP calibration graph was linear at low concentrations but showed a slight curvature at high concentrations (>100 μmol/l). A quadratic calibration curve was therefore used for evaluation of the concentrations. However, as the preliminary results indicated poor precision and accuracy at concentrations close to the LOQ for the quadratic curve, a weighted (1/C) linear calibration curve was used to evaluate low concentrations.

Over the range 0.6–17 μmol/l, six point standard curves, weighted by 1/C (where C=conc.), were calculated by the least squares linear regression method. To calculate higher concentrations a seven point quadratic standard curve over the concentration range 0.6–175 μmol/l was used.

2.4.2. Metabolite assay

Calibration standards were prepared by spiking blank human plasma to seven different concentrations in the range of 7–1200 nmol/l of EoM, 7–1200 nmol/l of EaM, 5–560 nmol/l of E1 and 2–110 nmol/l of E2, respectively. The standards were processed as described above. Calibration curves of EoM and EaM were established by a Millennium chromatography manager interfaced to the gas chromatograph. The peak-area ratios of EoM to PNU-212176 and EaM to PNU-210510 versus the concentrations of the substances in the calibration standards were plotted. The curves were evaluated by the least squares linear regression method, weighted by 1/C. The peak-area ratios of E1 to E1-D_4 and E2 to E2-D_4 were entered into a Microsoft Excel spreadsheet. The calibration curves were plotted and the concentrations were calculated using the same procedure as described above for EoM and EaM.

3. Results

3.1. Selectivity

EMP and the internal standard PNU-210462 gave well separated peaks in the chromatographic system used. Drug-free human plasma was tested for interference from endogenous components, and no interfering peaks were observed. Also EoM, EaM and the corresponding internal standards gave well separated peaks. There was no interference from the endogenous matrix. Chromatograms obtained for blank plasma and plasma spiked with EMP or the four metabolites respectively are shown in Fig. 2a–i.

3.2. Linearity

The EMP calibration graph over the range 0.6–175 μmol/l adapted smoothly to the quadratic equation. The C.V. ranged from 0.7% to 28% (at LOD, i.e. 0.6 μmol/l), maximum bias was −11.4% and the coefficient of determination (r^2) was 0.999 or greater. For the linear EMP calibration curve over the range 0.6–17 μmol/l the C.V. ranged from 0.8% to 11%, maximum bias was 6.7% and the coefficient of correlation (r) was 0.997 or greater.

The metabolite calibration curves were all linear and the coefficients of correlation (r) were 0.997 or greater for all compounds. For the EoM calibration curve over the concentration range 7–1200 nmol/l C.V. ranged from 2.8% to 16% (at 7 nmol/l) and maximum bias was −6.1%. For the EaM calibration curve over the concentration range 7–1200 nmol/l C.V. ranged from 1.8% to 7.2% and maximum bias was 3.2%. For the E1 calibration curve over the concentration range 5–560 nmol/l C.V. ranged from 1.3% to 14% and maximum bias was −12% (at 5 nmol/l). Finally, for the E2 calibration curve over the concentration range 2–110 nmol/l C.V. ranged from 2.1% to 11% and maximum bias was 4.7%.

3.3. Precision and accuracy

3.3.1. EMP

Human plasma was spiked with EMP to five different concentrations; ca. 2.3, 4.6, 6.9, 46 and 173 μmol/l. Aliquots of 1.0 ml were deep frozen and stored at −70°C. Duplicate plasma samples were analysed on 8 different occasions. The linear calibration curve was used to evaluate the samples of 2.3–6.9 μmol/l and the quadratic curve was used to evaluate the samples of 2.3 (as a comparison between the two curves), 46 and 173 μmol/l. The repeatability as coefficient of variation, C.V., ranged from 2.1 to 14.5%. The intermediate precision as coefficient of variation, C.V., ranged from 7.1 to 13.5 (18.6%). The accuracy ranged from 94.8 to 103.8 (109.1%). The bracketed C.V. for precision, 18.6%, and accuracy, 109.1%, was obtained for the 2.3 μmol/l sample, evaluated by the quadratic curve. The results are presented in Table 1.

3.3.2. Metabolites

Human plasma was spiked with a mixture of the metabolites to three different (low, medium and high) concentrations; ca. 95, 356 and 950 nmol/l of EoM, 96, 362 and 964 nmol/l of EaM, 35, 175 and 526 nmol/l of E1 and 22, 56 and 93 nmol/l of E2. Aliquots of 1.0 ml were deep frozen and stored at −70°C. Duplicate or triplicate plasma samples were analysed on seven different occasions. The repeatability, as coefficients of variation, ranged from 7.3 to 13.2% (low concentration), from 3.6 to 7.9% (medium concentration) and from 1.9 to 5.4% (high

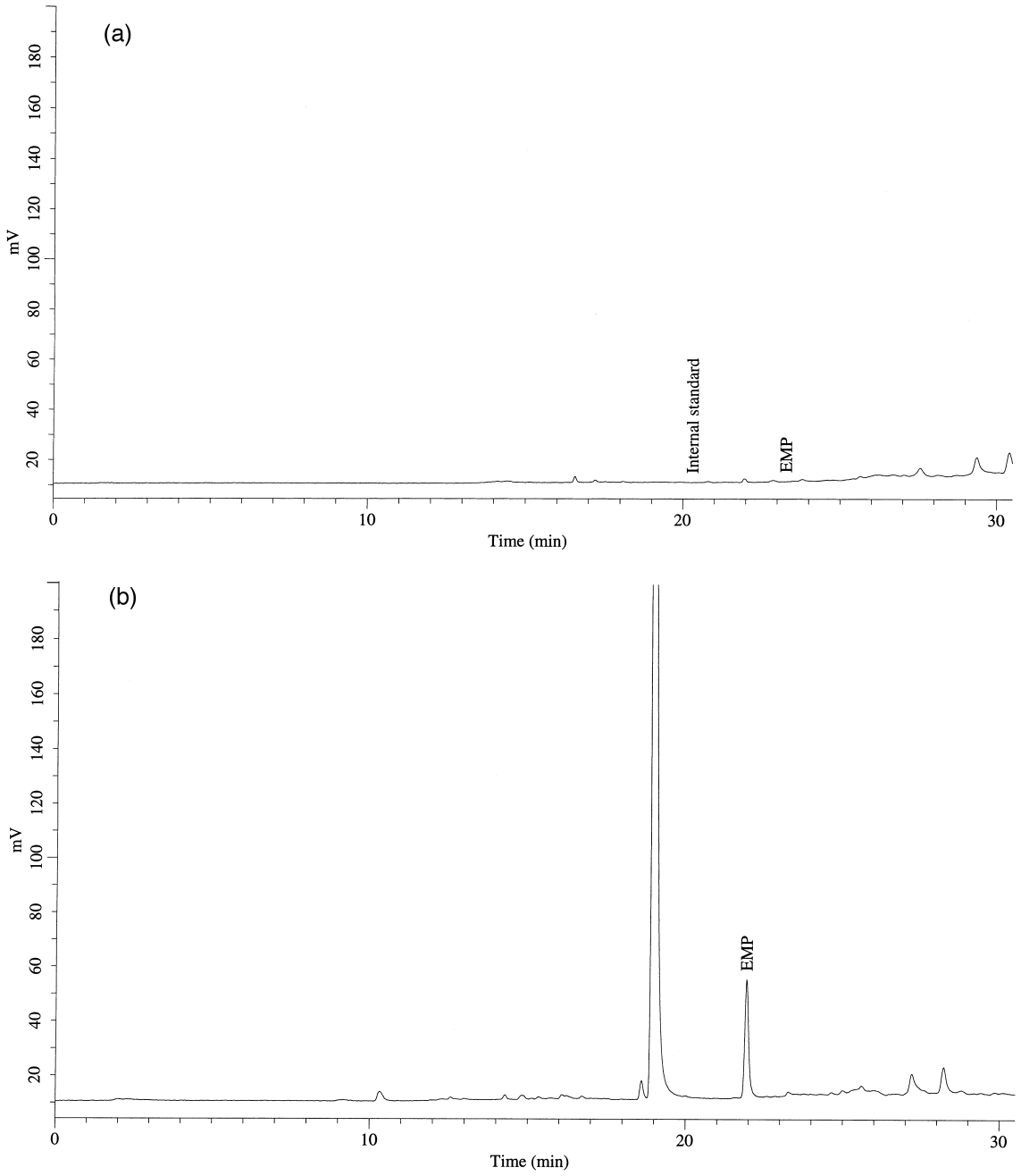


Fig. 2. (a) Blank plasma, estramustine phosphate assay; (b) estramustine phosphate (5.8 μmol/l spiked in blank plasma); (c) estramustine phosphate (46 μmol/l spiked in blank plasma); (d) blank plasma estromustine and estramustine assay; (e) estromustine (60 nmol/l) and estramustine (60 nmol/l) in spiked plasma; (f) estromustine (356 nmol/l) and estramustine (361 nmol/l) in spiked plasma; (g) blank plasma, estrone and estradiol assay (normalised peaks); (h) estrone (21.6 nmol/l) and estradiol (13.6 nmol/l) in spiked plasma (normalised peaks); (i) estrone (161 nmol/l) and estradiol (55.8 nmol/l) in spiked plasma (normalised peaks).

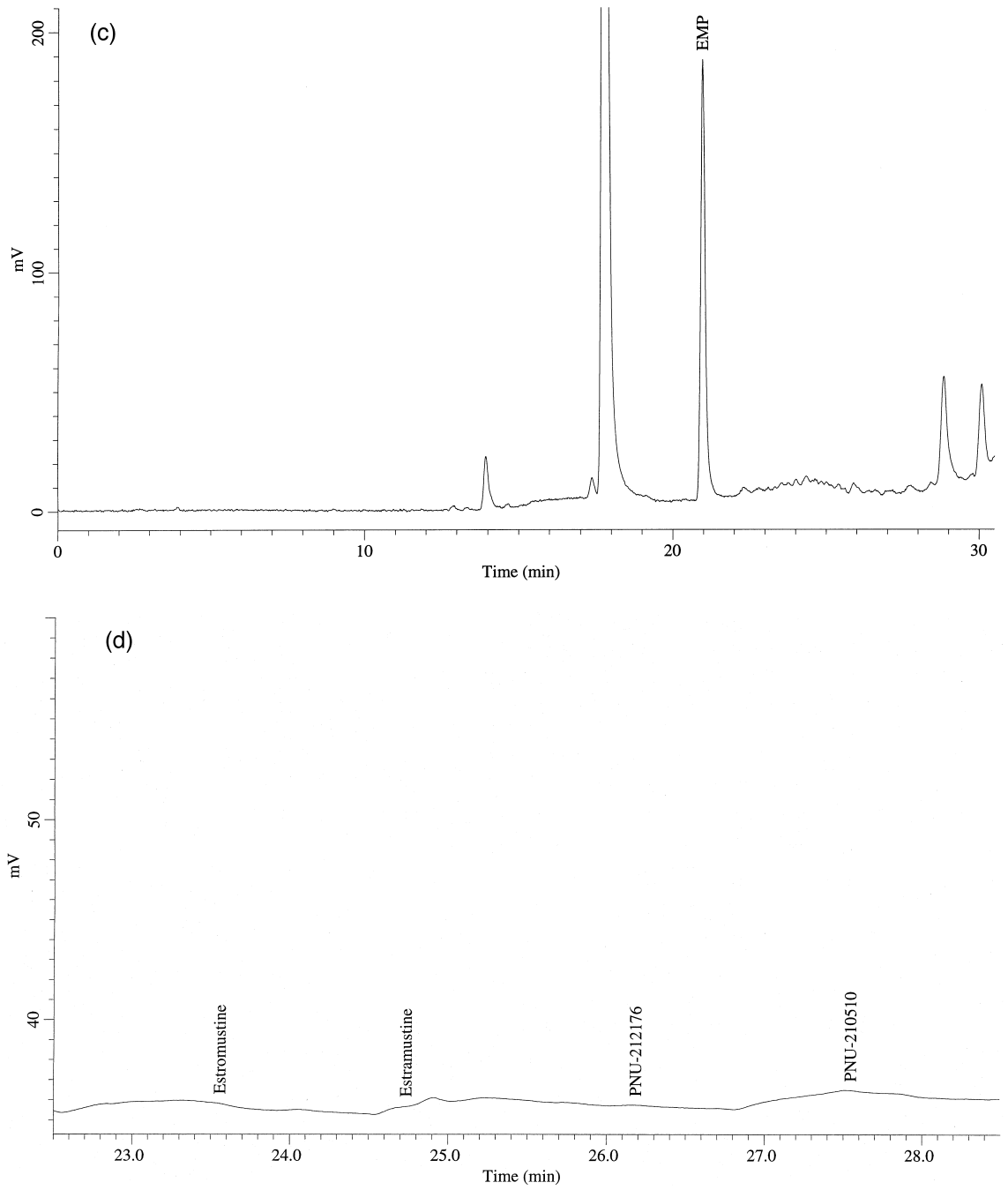
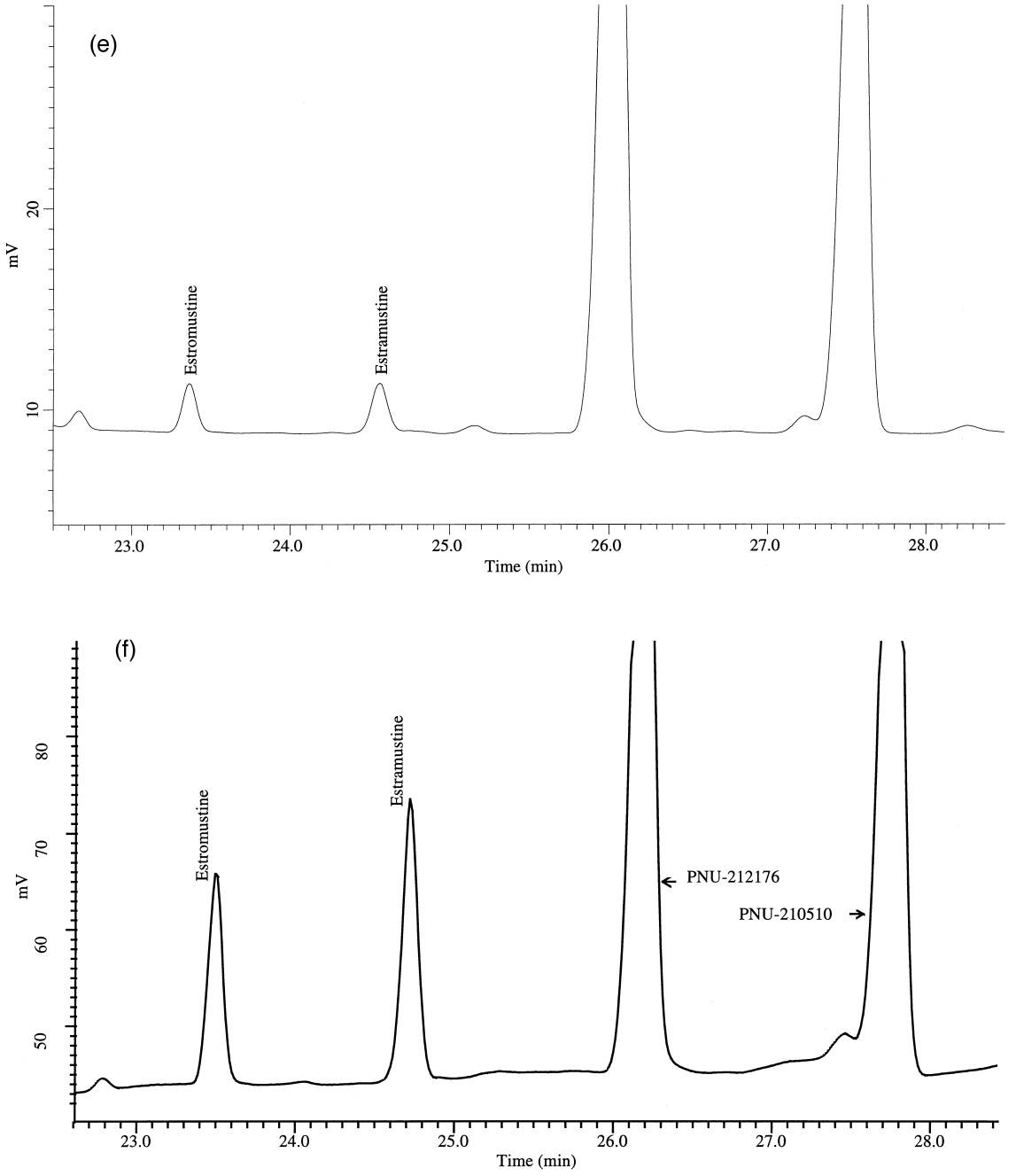


Fig. 2 (*continued*).

concentration). The intermediate precision as coefficients of variation ranged from 9.2 to 12.7% (low concentration), from 6.2 to 10.1% (medium concentration) and from 6.4 to 11.3% (high concentration). The accuracy ranged from 92.5 to 99.9% (low concentration), from 91.8 to 103.9% (medium



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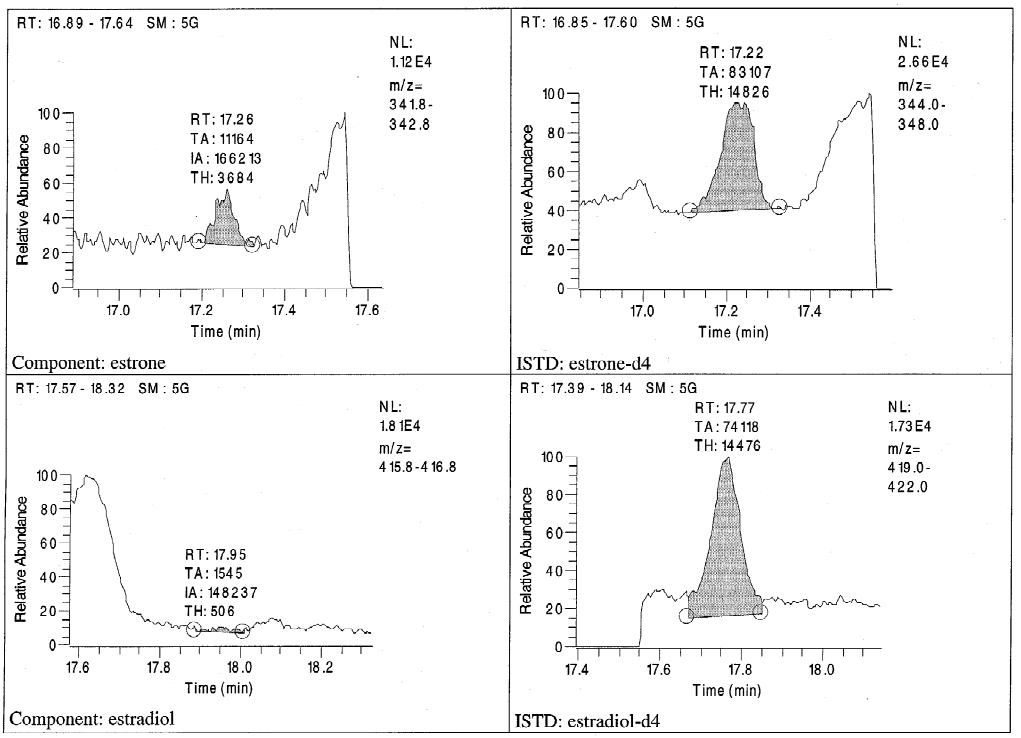

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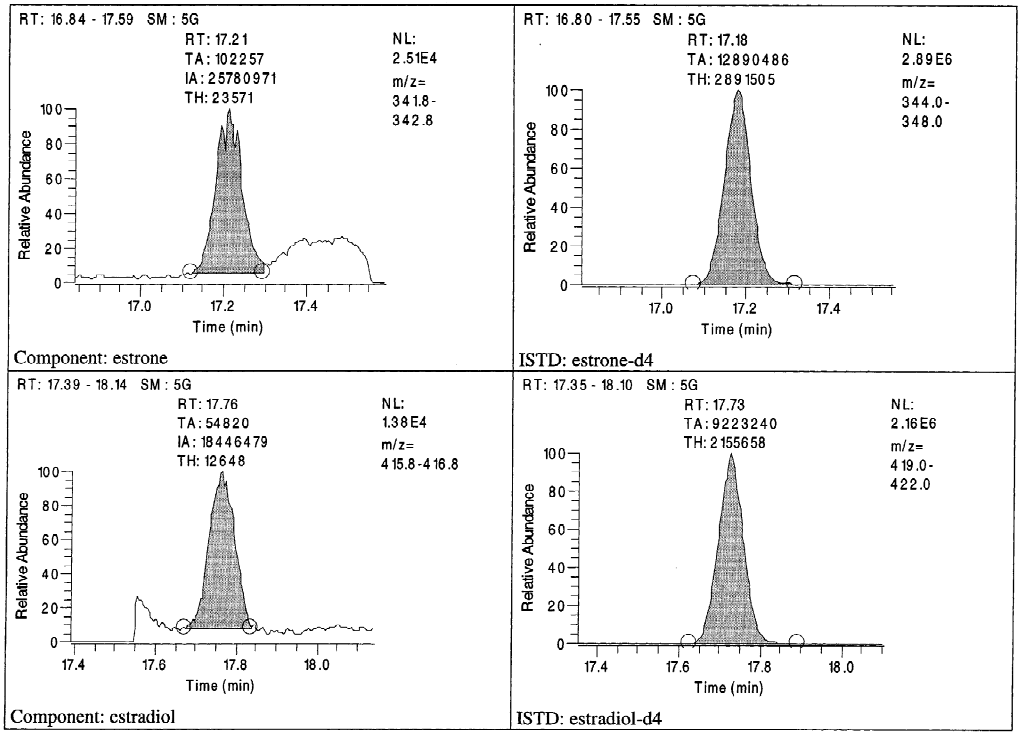

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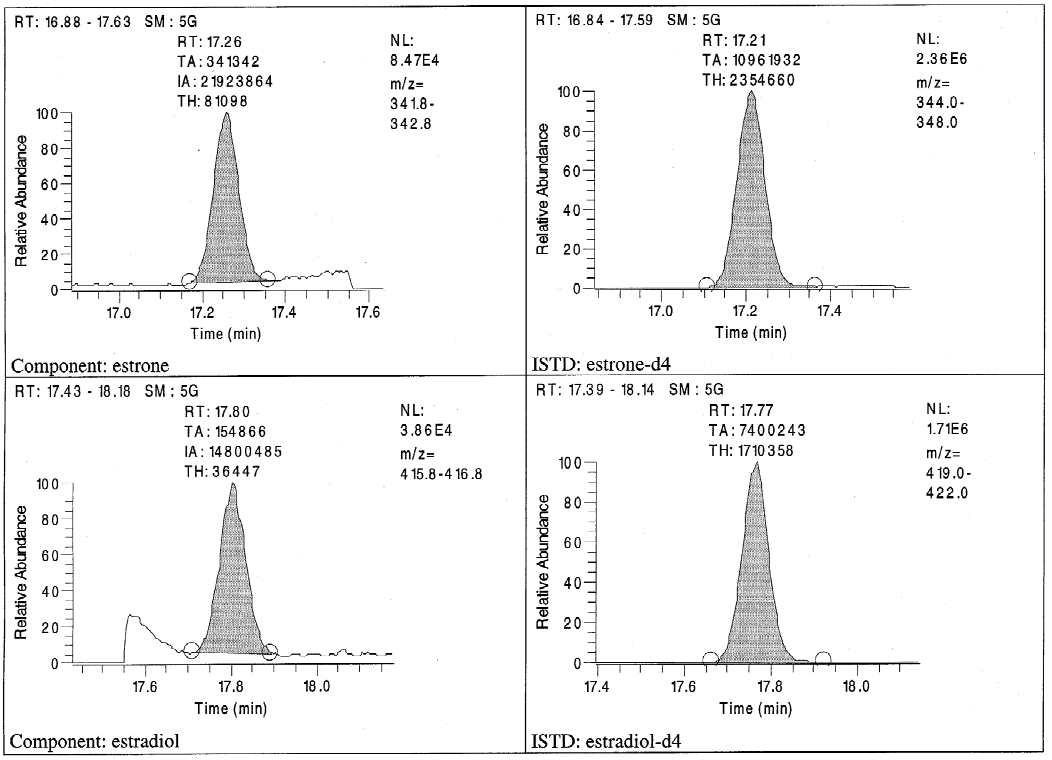

Fig. 2 (*continued*).

concentration) and from 93.6 to 102.7% (high concentration). The results are presented in Table 2.

3.4. Limit of detection

To determine the limit of detection (LOD), 1.0 ml of blank human plasma was analysed at 8–12 different occasions. The apparent concentrations detected at the retention time of the analytes were calculated. The LOD was defined as M+t×SD, where M was the mean and SD was the standard deviation of the apparent concentrations obtained in blank plasma, t was the t-distribution at the 99% level.

Table 1
Precision and accuracy of estramustine phosphate

Calibration curve	Nominal concentration (μmol/l)	Found concentration (mean) (μmol/l)	Repeatability (C.V.%)	Intermediate precision (C.V.%)	Accuracy (%)	Number (*n*)
Linear	2.30	2.39	14.5	13.5	103.8	16
Linear	4.61	4.58	9.1	7.7	99.3	16
Linear	6.92	6.67	7.1	7.9	96.4	16
Quadratic	2.30	2.51	12.6	18.6	109.1	16
Quadratic	46.2	43.7	2.1	9.1	94.8	16
Quadratic	173	171	4.4	7.1	98.9	16

Table 2
Precision and accuracy of the metabolites of estramustine phosphate

Substance	Nominal concentration (nmol/l)	Found concentration (mean) (nmol/l)	Repeatability (C.V.%)	Intermediate precision (C.V.%)	Accuracy (%)	Number (*n*)
Estromustine	95.0	94.9	10.8	10.9	99.9	16
Estromustine	356	359	6.1	6.2	100.6	16
Estromustine	950	949	5.4	6.8	99.9	16
Estramustine	96.4	93.1	7.3	9.2	96.6	16
Estramustine	362	376	7.9	8.1	103.9	16
Estramustine	964	990	3.7	6.4	102.7	16
Estrone	35.1	34.5	13.2	12.7	98.5	16
Estrone	175	171	3.6	6.6	97.4	16
Estrone	526	503	1.9	6.4	95.7	16
Estradiol	22.2	20.5	9.0	10.6	92.5	16
Estradiol	55.5	50.9	4.5	10.1	91.8	16
Estradiol	92.5	86.6	4.6	11.3	93.6	16

The limits of detection were 0.6 μmol/l for EMP, 7 nmol/l for EoM, 7 nmol/l for EaM, 5 nmol/l for E1 and 2 nmol/l for E2.

3.5. Limit of quantitation

The limit of quantitation (LOQ) was defined as the lowest concentration with a precision <20%, an accuracy of 80–120% and with a signal that significantly differed from that of the limit of detection.

The LOQ values were 2.3 μmol/l for EMP, 30 nmol/l for EoM, 30 nmol/l for EaM, 12 nmol/l for E1 and 8 nmol/l for E2.

3.6. Recovery

The recovery of EMP was determined by spiking plasma to low, medium and high concentrations and processing them in accordance with the method. The peak areas of the prepared plasma samples were compared with peak areas of the corresponding volumes and concentrations of standard solutions which were just evaporated to dryness, dissolved in corresponding volumes of methanol and injected onto the column. The recovery of the sample preparation procedure was 70%.

The recoveries of the metabolites were determined by spiking plasma to concentrations throughout the calibrated ranges and processing them in accordance with the method. The peak areas of the prepared plasma samples were compared with peak areas of the corresponding volumes and concentrations of standard solutions which were just evaporated to dryness, derivatized, dissolved in corresponding volumes of xylene/toluene and injected onto the columns. The found recoveries ranged from 70 to 90%.

3.7. Stability

The stability of spiked plasma samples stored at −70°C was studied for all compounds over 2.5–4 years. The stability of EMP in plasma samples was four years when stored at −70°C, the compound degradation was less than 5% during the period. The metabolites were stable for two years in plasma samples when stored at −70°C. The concentration changes of estromustine and estrone were less than 5% and the changes in concentration of estramustine and estradiol were less than 15% during the period.

3.8. Applications

The methods are presently used in the analysis of samples from clinical trials [17–21]. A typical plasma profile of EMP and its metabolites is shown in Fig. 3.

Table 3
Assay for estramustine in human plasma-comparison of different techniques

	HPLC Normal phase [6]	GC	LC–MS–MS[a] (Data not published)
Sample preparation	Solvent extraction	SPE/Solvent extraction	Protein precipitation
Derivatisation	N/A[b]	Silanization	N/A[b]
Detection	Fluorescence	NPD	MS–MS
LOD	90 nmol/l	7 nmol/l	23 nmol/l
Sample throughput	40–50 samples/day	40–50 samples/day	100–200 samples/day

[a] Ionisation was performed at atmospheric pressure (positive ion ionspray) on an API Sciex 365 instrument. EaM was separated from EoM and EMP on a Waters symmetry RP-8, 3 μm, 50×4.6 mm column. The mobile phase was methanol: 10 m*M* ammonium acetate (pH 4.0) (80:20, v/v).
[b] Not applicable.

4. Conclusions

There is an obvious need for further determination of EMP and its metabolites in pharmacokinetic studies: EMP is currently undergoing extended clinical trials against prostrate cancer as well as against other forms of cancer [17–21]. Furthermore, after oral administration there is an interaction between EMP and polyvalent cations such as calcium in the food, which reduces the extent of absorbtion of EMP [22] and pharmacokinetic studies with alternative formulations to optimise treatment of cancer patients with EMP are ongoing.

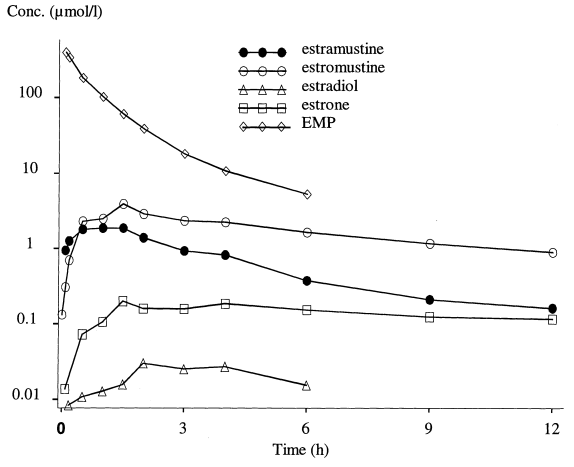

Fig. 3. Serum concentration–time profiles after single intravenous administration of 900 mg of Estracyt to a patient.

The methods presented in this paper have been found to perform well. The LOD of the metabolite method is lowered to one tenth for EoM- and EaM and to 50% for the estrogens. Furthermore, the sample throughput has been improved and is now three times as high as for the formerly used method [14]. So far, it has not been possible to achieve as low LOQs for EoM and EaM with any other technique. An investigation of the possibility of using LC–MS–MS for the determinations has been made. However, it has not been possible to achieve as low LOQ for the metabolites with the LC–MS–MS technique as with the presently used GC–NPD and GC–MS techniques (data not published), Table 3.

In conclusion; the described procedures provide good linearity, selectivity, precision and accuracy for the determination of EMP as well as of EoM, EaM, E1 and E2 in plasma. The methods are therefore suitable for pharmacokinetic and clinical studies. Furthermore, compared to the previous metabolite method, the sensitivity has been significantly lowered and the sample throughput greatly improved.

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