LC–MS/MS coupled with immunoaffinity extraction for determination of estrone, 17β-estradiol and estrone 3-sulfate in human plasma☆

Jun Hosogi [a,*], Hideyuki Tanaka [a], Kazuhiro Fujita [a], Takashi Kuwabara [a], Shigeo Ikegawa [b], Norihiro Kobayashi [c], Nariyasu Mano [d,e], Junichi Goto [d]

[a] Pharmacokinetic Research Laboratories, Research Division, Kyowa Hakko Kirin Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Sizuoka 411-8731, Japan
[b] Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan
[c] Kobe Pharmaceutical University, 4-19-1 Motoyama-kitamachi, Higashinada-ku, Kobe 658-8558, Japan
[d] Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
[e] Laboratory of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

ARTICLE INFO

ABSTRACT

Determination of estrogens in plasma is important in evaluation of effects of some anticancer drugs, such as aromatase inhibitors. However, as reported previously, high performance liquid chromatography–radio immunoassay (HPLC–RIA) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) with chemical derivatization require complicated sample preparation. In this study, a highly sensitive and simple method for determination of estrone (E1), 17β-estradiol (E2) and estrone 3-sulfate (E1S) in human plasma has been developed. Following diethylether extraction from plasma, analytes were purified by immunosorbents and then determined by LC–MS/MS using electrospray ionization (ESI). Immunosorbents were prepared by immobilization of specific antibodies raised against each analyte onto solid support. Use of selective immunosorbents in sample preparation removed interference in plasma samples that would cause ionization suppression, and markedly improved the sensitivity of LC–MS/MS for these analytes, without derivatization. Calibration curves of each analyte showed good linearity and reproducibility over the range of 0.05–50 pg/injection for E1, 0.2–50 pg/injection for E2 and 0.05–300 pg/injection for E1S, respectively. The mean values of lower limits of quantification (LLOQ) in human plasma corrected by recovery of deuterated estrogens (internal standard, I.S.) were 0.1892 pg/mL for E1, 0.7064 pg/mL for E2 and 0.3333 pg/mL for E1S, respectively. These LLOQ values were comparable to those previous reported using HPLC–RIA and LC–MS/MS. Using this method, the normal levels of three estrogens in healthy female plasma (n = 5) were determined. The mean values of E1, E2 and E1S were 38.0 pg/mL (range 24.8–53.0), 34.3 pg/mL (22.6–46.6) and 786 pg/mL (163–2080), respectively. The immunoaffinity LC–MS/MS described here allows sensitive and accurate quantification of E1, E2 and E1S without laborious sample preparation.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

More than 60% of human breast cancers express estrogen receptor [1,2] and these tumors often require estrogens for their growth. Plasma estrogen level is also associated with the risk of breast cancer [3,4]. Therefore, estrogen deprivation is an effective action of treatment in hormone sensitive breast cancer. Estrogen synthesis through aromatase and steroid sulfatase in peripheral tissue and tumor are probably important for tumor growth in postmenopausal women. Thus, these enzymes are among the therapeutic targets of hormone sensitive breast cancer and several aromatase inhibitors have already succeeded [5–7]. Therefore, determination of plasma estrogen levels is important in evaluation of effects of aromatase inhibitors. Plasma estrogen levels are very low, and thus sensitive analytical techniques are required to determine the change in estrogen levels. A number of immunoassay have been reported for estrogen determination in plasma and tumor [8–10]. Although HPLC–RIA or RIA are highly sensitive methods in current use, the process could suffer from complicated preparation and cross-reaction with other steroids in plasma and tissue. LC–MS/MS with solid phase extraction have been used for the determination of estrogens in wastewater [11,12]; however, quantitation limits of these methods are typically above 1 ng/mL, insufficient for estrogen determination in human plasma. Moreover, their purification by HPLC combined with solid phase extraction is insufficient, because
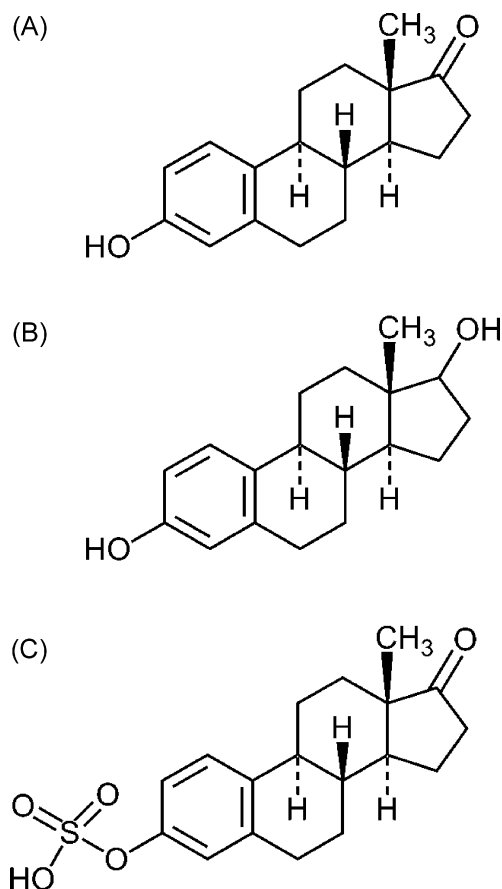
Fig. 1. Chemical structures of (A) estrone (E1), (B) 17β-estradiol (E2) and (C) estrone 3-sulfate (E1S).

complex extract matrix in plasma frequently affects electrospray ionization process of estrogens, greatly reducing the sensitivity. Recently, several LC–MS/MS using derivatization were developed to determine E1 and E2 in plasma [13–15]. These methods are highly sensitive and specific, but require laborious sample preparation.

In this study, we have developed a highly sensitive LC–MS/MS coupled with immunoaffinity extraction for direct determination of estrone, 17β-estradiol and estrone 3-sulfate in human plasma and measured three estrogen levels in plasma from healthy women.

2. Experimental

2.1. Chemicals and reagents

Estrone and 17β-estradiol (Fig. 1A and B) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Estrone 3-sulfate (Fig. 1C) was synthesized at Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). Estrone-2,4,16,16-d_4 (E1-d_4), 17-β-estradiol-16,16,17-d_3 (E2-d_3) and estrone-2,4,16,16-d_4 3-sulfate sodium salt (E1S-d_4) were purchased from Aldrich Chemical (Milwaukee, WI, USA). Acetonitrile and methanol were of HPLC grade from Kanto Chemical (Tokyo, Japan). Purified water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The other reagents were of special grade and commercially available. The three estrogens and three deuterated estrogens were separately dissolved at a concentration of 200 μg/mL in methanol and each solution was stored at −20 °C.

2.2. Human plasma

Plasma samples from five healthy women were purchased from Scantibodies Laboratory (n = 5, Santee, CA, USA). For validation, five plasma samples were pooled. All plasma samples were stored at −80 °C until analysis.

2.3. Immunosorbents

Two immunosorbents (Column A and Column B) were prepared by immobilization of specific antibodies against each analyte onto solid support. Column A was used for extraction of E1 and E2, and Column B was used for extraction of E1S. Purification of IgG fraction and binding reaction to solid support basically followed the previous report [16]. Twenty mL of each antiserum raised against estrone and 17β-estradiol (for Column A, obtained from rabbits following injection of estrone 17-(O-carboxymethyl)oxime-bovine serum albumin conjugate, synthesized at Kyowa Hakko Kirin), and against estrone 3-sulfate (for Column B, obtained from rabbits following injection of estrone 3-carboxymethyl ether-bovine serum albumin conjugate, synthesized at Tohoku University, Sendai, Japan) were added to 70 mL of 0.4 w/v% Rivanol (6,9-diamino-2-ethoxyacridine lactate, Nacalai tesque, Kyoto, Japan) and stirred gently on ice for 60 min. The precipitate was removed by centrifugation (approximately $2000 \times g$, 10 min, 4 °C) and small molecular size fraction was trapped by 2.0 g of activated charcoal. The supernatant containing IgG fraction was concentrated using Centriprep YM-10 filters (Millipore) and then dialyzed (10,000 MWCO cellulose membrane) twice overnight against 500 mL of 0.1 mmol/L 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 8.0). Affigel-10 (Bio-Rad, Hercules, CA, USA), agarose gel solid support, was washed with 50 mL of isopropyl alcohol, 100 mL of 10 mmol/L sodium acetate (pH 4.5) and 50 mL of 0.1 mmol/L HEPES buffer. Each dialyzed IgG fraction was diluted to 15 mL with 0.1 mmol/L HEPES buffer and was then added to 10 mL of washed Affigel-10. IgG fraction was allowed to bind to the solid support for 24 h at 4 °C, with gentle agitation. To cap remaining activated ester on the solid support, 0.75 mL of 1 mol/L ethanolamine (pH 8.0) was added and agitated for 24 h at 4 °C. After completion of the reaction, the sorbents were washed with 50 mL of 0.1 mmol/L HEPES buffer and 50 mL of 50 mmol/L phosphate buffer (pH 7.3). Each immunosorbent was filled in 12 Poly-prep columns (Bio-Rad) and stored sealed in 50 mmol/L phosphate buffer (pH 7.3) containing 0.2 w/v% of sodium azide at 4 °C.

2.4. Plasma sample preparation

Immunosorbents were pre-conditioned before use first with 10 mL of water, then 8 mL of 95 vol% methanol, then 10 mL of water and 8 mL of 50 mmol/L phosphate buffer (pH 7.3), in that order. 50 μL of 50 vol% methanol containing 0.4 ng/mL three deuterated estrogens (I.S.) was added to human plasma (1 mL) to correct the overall recovery and then diluted with 1 mL of McIlvaine's citric acid-phosphate buffer (pH 4.0). After diethylether extraction (3 mL × 2), organic layer containing E1 and E2 was dried under a stream of nitrogen. The residue was reconstituted with 100 μL of ethanol and diluted with 1.9 mL of 50 mmol/L phosphate buffer (pH 7.3) containing 0.05 vol% Tween 80. The water layer containing E1S was diluted with 1 mL of 50 mmol/L phosphate buffer (pH 7.3) containing 0.05 vol% Tween 80. The organic layer was applied to the immunosorbent against estrone and 17β-estradiol (Column A), and the water layer was applied to the immunosorbent against estrone 3-sulfate (Column B). After washing with 10 mL of water, estrogens were eluted with 5 mL of 95 vol% methanol from immunosorbents and then dried under a stream of nitrogen. The residue was recon-

Table 1
Precision and accuracy of determination of E1, E2 and E1S in methanol solution.

Study	Compound	Injected amount (pg/injection)	Found amount[a] (pg/injection)	Precision[b] (%)	Accuracy[c] (%)
Intra-day[d]	E1	0.05	0.05874 ± 0.00339	5.8	17.5
		1	1.029 ± 0.024	2.3	2.9
		50	50.94 ± 0.41	0.8	1.9
	E2	0.2	0.2055 ± 0.0267	13.0	2.7
		2.5	2.589 ± 0.118	4.6	3.6
		50	50.33 ± 0.66	1.3	0.7
	E1S	0.05	0.04978 ± 0.00136	2.7	−0.4
		5	5.088 ± 0.128	2.5	1.8
		300	281.3 ± 1.3	0.5	−6.2
Inter-day[e]	E1	0.05	0.05387 ± 0.00656	12.2	7.7
		1	1.038 ± 0.019	1.8	3.8
		50	50.98 ± 0.53	1.0	2.0
	E2	0.2	0.2280 ± 0.0335	14.7	14.0
		2.5	2.578 ± 0.120	4.7	3.1
		50	50.67 ± 0.83	1.6	1.3
	E1S	0.05	0.04830 ± 0.00513	10.6	−3.4
		5	5.118 ± 0.085	1.7	2.4
		300	292.4 ± 8.3	2.8	−2.5

[a] Mean ± S.D.
[b] Coefficient of variation.
[c] Relative error.
[d] $n = 5$.
[e] $n = 15$.

stituted with 200 μL of mobile phase and 100 μL was injected to LC–MS/MS.

2.5. HPLC and MS/MS conditions

In the case of E1 and E2 determination, the HPLC system consisted of two G1312A binary pumps (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA), a G1379A degasser, a G1316A column component, an HTC-PAL auto sampler (CTC Analytics, Zwingen, Switzerland) and a syringe pump Model 22 (Harvard

Apparatus, Holliston, MA, USA, for postcolumn addition). Analysis was carried out with a column switching technique. A Symmetry C18, 3.5 μm, 2.1 mm i.d. × 10 mm (Waters, Milford, MA, USA) as trapping column and an XTerra Phenyl, 5 μm, 2.1 mm i.d. × 150 mm (Waters) as analytical column were used for chromatographic separation. The initial composition of Pump 1 (to trapping column) was 10 vol% of methanol at a flow rate of 0.2 mL/min and that of Pump 2 was 80 vol% of methanol at a flow rate of 0.15 mL/min. The time program of column switching valve was as follows: 0–3 and 9–18 min, valve position A (to trapping column); 3–9 min, valve position B
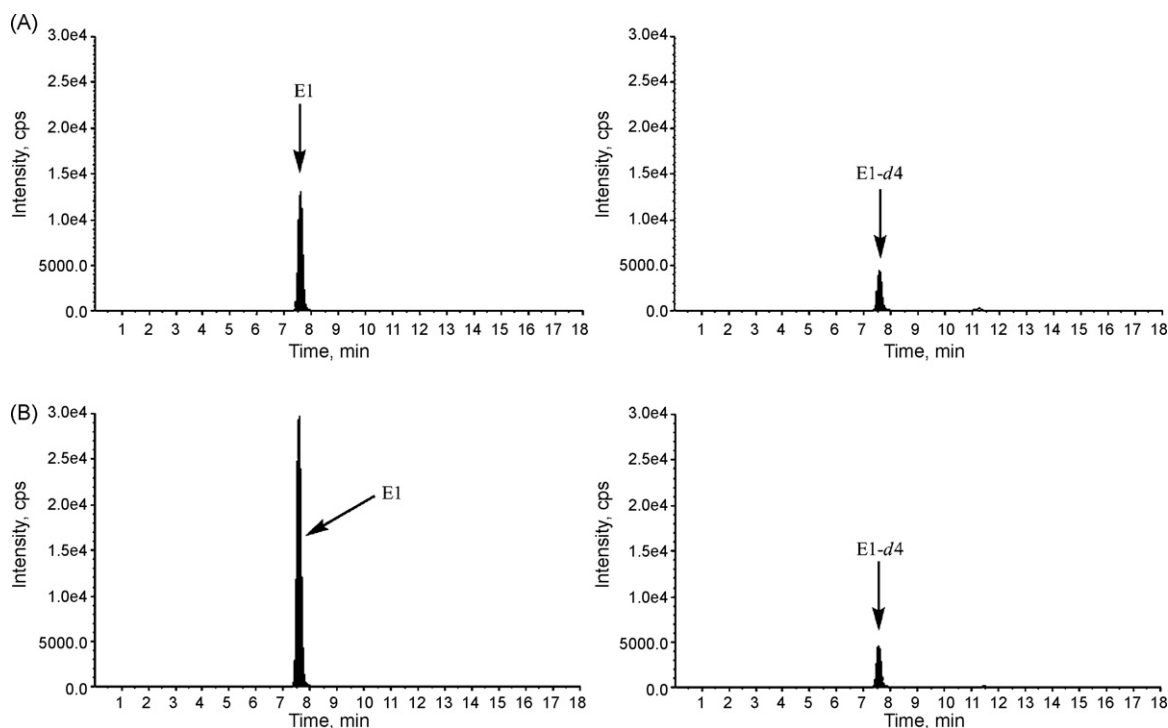
Fig. 2. Typical MRM chromatograms for E1 (Q1/Q3 269.15/145.07) and E1-d_4 (I.S., Q1/Q3, 273.18/147.08) in extracted plasma after the immunoaffinity extraction. (A) The human plasma spiked with 20 pg/mL of E1-d_4 (I.S.), E2-d_3 (I.S.) and E1S-d_4 (I.S.). (B) The human plasma spiked with 50 pg/mL of E1, E2 and 300 pg/mL of E1S, and 20 pg/mL of E1-d_4 (I.S.), E2-d_3 (I.S.) and E1S-d_4 (I.S.).
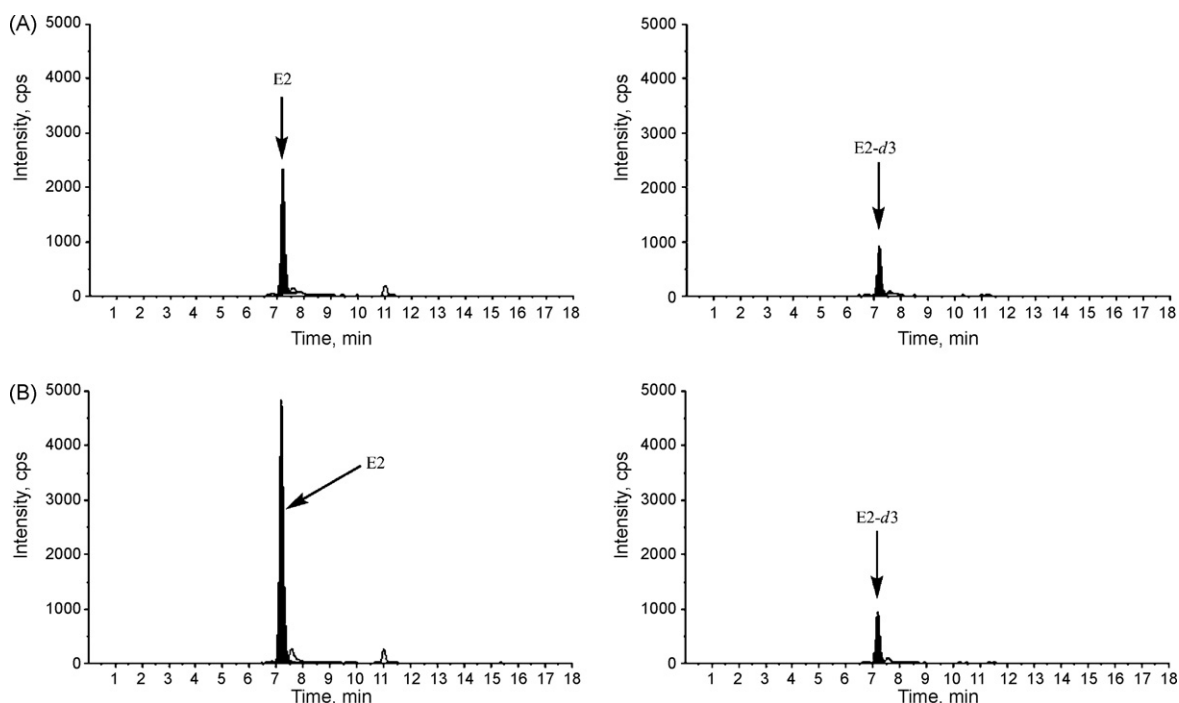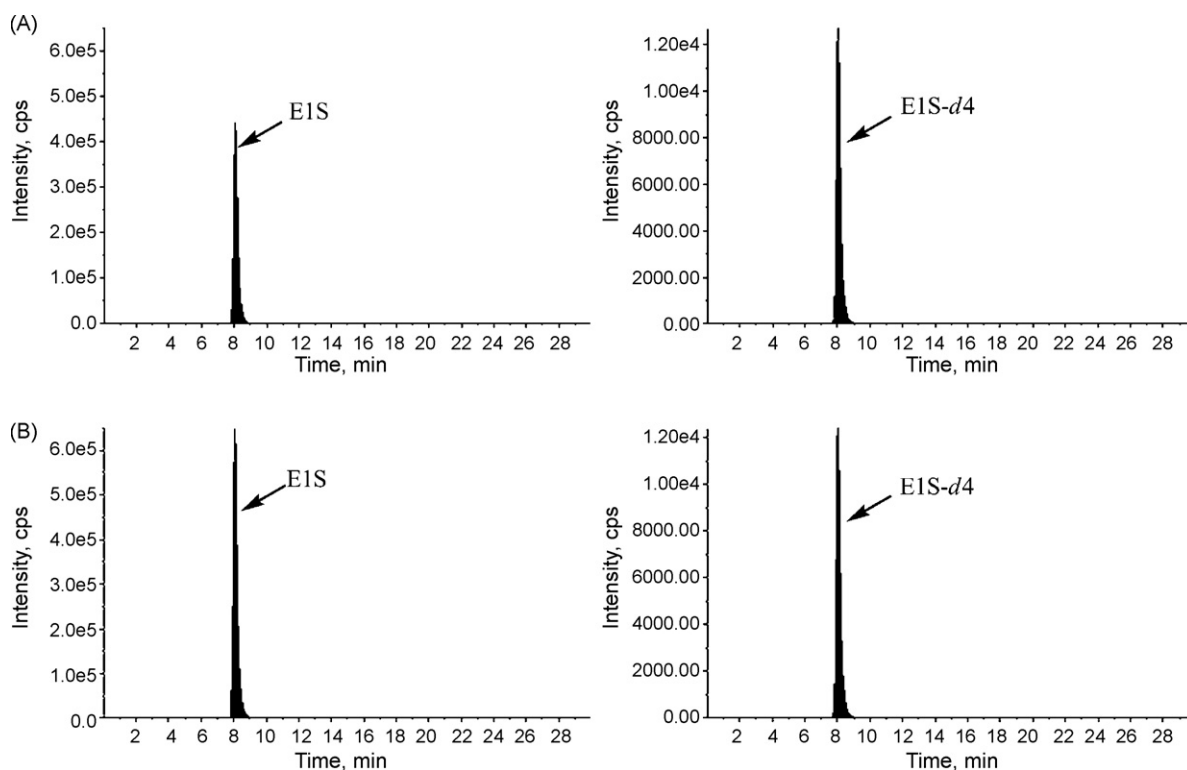
Fig. 3. Typical MRM chromatograms for E2 (Q1/Q3 271.17/145.07) and E2-d_3 (I.S., Q1/Q3 274.19/145.07) in extracted plasma after the immunoaffinity extraction. (A) The human plasma spiked with E1-d_4 (I.S., 20 pg/mL), E2-d_3 (I.S., 20 pg/mL) and E1S-d_4 (I.S., 20 pg/mL). (B) The human plasma spiked with E1 (50 pg/mL), E2 (50 pg/mL) and E1S (300 pg/mL), and E1-d_4 (I.S., 20 pg/mL), E2-d_3 (I.S., 20 pg/mL) and E1S-d_4 (I.S., 20 pg/mL).

(to MS/MS). The columns were washed with methanol over 3 min before returning initial condition. As postcolumn-addition modifier, methanol with 32 mmol/L NH$_3$ was added to mobile phase at a flow rate of 2.5 μL/min [17].

In the case of E1S determination, the HPLC system consisted of a G1312A binary pump, a G1379A degasser, a G1316A column component, and an HTC-PAL auto sampler. A Symmetry C18 (3.5 μm, 2.1 mm i.d. × 150 mm (Waters)) was used for chromatographic sep-

Fig. 4. Typical MRM chromatograms for E1S (Q1/Q3 349.11/269.15) and E1S-d_4 (I.S., Q1/Q3 353.14/273.18) in extracted plasma after the immunoaffinity extraction. (A) The human plasma spiked with E1-d_4 (I.S., 20 pg/mL), E2-d_3 (I.S., 20 pg/mL) and E1S-d_4 (I.S., 20 pg/mL). (B) The human plasma spiked with E1 (50 pg/mL), E2 (50 pg/mL) and E1S (300 pg/mL), and E1-d_4 (I.S., 20 pg/mL), E2-d_3 (I.S., 20 pg/mL) and E1S-d_4 (I.S., 20 pg/mL).

226

J. Hosogi et al. / J. Chromatogr. B 878 (2010) 222–227

Table 2
Precision and accuracy of determination of E1, E2 and E1S in human plasma.

Study	Compound	Added conc.[a] (pg/mL)	Found conc.[a] (pg/mL)	Calculated conc.[b] (pg/mL)	Precision[c] (%)	Accuracy[d] (%)
Intra-day[e]	E1	–[f]	38.41 ± 0.55	–	1.4	–
		50	86.02 ± 1.18	47.61 ± 1.18	1.4	−4.8
	E2	–[f]	43.47 ± 2.41	–	5.5	–
		50	95.53 ± 4.50	52.06 ± 4.50	4.7	4.1
	E1S	–[f]	588.2 ± 3.0	–	0.5	–
		300	897.3 ± 3.7	309.1 ± 3.7	0.4	3.0
Inter-day[g]	E1	–[f]	38.83 ± 1.39	–	3.6	–
		50	85.93 ± 2.11	47.10 ± 2.11	2.5	−5.8
	E2	–[f]	44.02 ± 2.12	–	4.8	–
		50	93.95 ± 3.52	49.93 ± 3.52	3.7	−0.1
	E1S	–[f]	564.2 ± 22.4	–	4.0	–
		300	873.7 ± 33.4	309.5 ± 33.4	3.8	3.2

[a] Mean ± S.D.
[b] Found conc. – (mean of found blank conc.), mean ± S.D.
[c] Coefficient of variation.
[d] Relative error.
[e] $n = 5$.
[f] Blank sample.
[g] $n = 15$.

aration. As mobile phase, Solvent A (5 mmol/L ammonium acetate (pH 5.4)–methanol (90:10, v/v)) and Solvent B (5 mmol/L ammonium acetate (pH 5.4)–methanol (10:90, v/v)) were used. The gradient was begun at 55% of Solvent B followed by a linear increase to 85% of Solvent B in 8 min and then to 100% in 1 min at a flow rate of 0.15 mL/min. The column was washed with Solvent B for 6 min before returning to initial conditions. As postcolumn-addition modifier, 2-(2-methoxy)ethoxyethanol was added into the mobile phase at a flow rate of 20 μL/min [18].

A triple quadrupole mass spectrometer API4000 (Applied Biosystems/MDS SCIEX, Concord, Canada) with ESI ion source was operated in negative mode. Multiple reaction monitoring (MRM) was chosen for quantitation and each tuning parameter was optimized for different transition pairs to enhance the sensitivity for ions. Precursor-product ion combinations of m/z 269.15/145.07 (Q1/Q3) for E1, 271.17/145.07 for E2, 349.11/269.15 for E1S, 273.18/147.08 for E1-d_4, 274.19/145.07 for E2-d_3 and 353.14/273.18 for E1S-d_4 were used.

2.6. Calibration curve

Human plasma was not used for calibration curve samples, as it contained endogenous estrogens and removal treatment of these compounds affected the matrix effects. Consequently, samples for calibration curve were prepared from methanol solution and the quantitation range in each plasma sample was corrected by the recovery of deuterated estrogens. Calibration curves were obtained from the peak area ratio (y) of analyte to I.S. against the concentrations of analyte (x) using Analyst Software Version 1.3.1 (Applied Biosystems/MDS SCIEX) (weight: $1/y^2$).

3. Results and discussion

The necessity of interpreting the pharmacological efficacy and toxicity of some anticancer drugs, such as aromatase inhibitors, is

resulting in increased demand for faster and better analytical methods for determination of estrogen. In this study, we have developed a highly sensitive assay for determination of three estrogens in human plasma, based on LC–MS/MS and immunoaffinity extraction.

The chromatographic separation of E1 and E2 was performed using a reversed-phase column and column switching technique. The ionization efficiencies of E1 and E2 were very low, and their ionization processes were susceptible to endogenous substances in plasma; thus, ammonia as postcolumn pH modifier was added to mobile phase to improve the ionization efficiency and stabilize the ionization conditions in the MS source. The ion intensities of E1 and E2 were enhanced 3-fold (data not shown).

The chromatographic separation of E1S was performed using a reversed-phase column with acidified mobile phase containing buffer solution, because it results in excellent chromatographic performance with good reproducibility [19]. 2-(2-Methoxy)ethoxyethanol (2-MEE) was added into the mobile phase at a flow rate of 20 μL/min to reduce ionization suppression caused by competition between the analyte and coexisting anions and disturbing fine droplet formation caused by high surface tension of mobile phase. 2-MEE improved the ionization process in the electrosprayed droplet and enhanced the ion intensity of E1S more than 5-fold (data not shown).

First, we checked reliability and sensitivity of the LC–MS/MS system in determination of three estrogens by methanol solution. Calibration curves of each analyte showed good linearity in the range of 0.05–50 pg/injection for E1, 0.2–50 pg/injection for E2 and 0.05–300 pg/injection for E1S, respectively. The signal-to-noise ratio (S/N) of the lower limits of calibration curve was 25.2 for E1, 36.4 for E2 and 15.4 for E1S, respectively. The intra-day ($n = 5$) and inter-day ($n = 3$) assay precisions in methanol solution were within 14.7%, and the accuracies were within ±17.5% at low concentration and within ±6.2% at middle and high concentrations (Table 1).

Table 3
Concentration of E1, E2 and E1S in human plasma.

Compound	Found conc. (pg/mL)					Mean (pg/mL)	S.D.
	1	2	3	4	5		
E1	53.0	37.6	29.0	45.6	24.8	38.0	11.6
E2	35.4	46.6	23.4	43.6	22.6	34.3	11.1
E1S	2080	697	340	651	163	786	756

In the next step, we used the same LC–MS/MS system for determination in human plasma. However, a major problem concerning plasma sample was ionization suppression caused by endogenous substance in plasma. Thus, to overcome ionization suppression selective immunosorbents were used in sample preparation prior to analysis.

Typical MRM chromatograms of a human blank plasma and plasma sample spiked with estrogens are shown in Figs. 2–4, in which no interference was observed. Overall recovery including ionization suppression in the electrospray process was calculated by comparing the peak area of deuterated estrogen (I.S.) in methanol solution to that of plasma sample. The recovery values from plasma sample were found to be 45.2–62.5% for E1, 47.3–69.1% for E2 and 24.6–34.4% for E1S, respectively. The mean values of lower limits of quantification (LLOQ) in plasma corrected by recovery of deuterated estrogens were 0.1892 pg/mL (range of 0.1600–0.2212) for E1, 0.7064 pg/mL (0.5789–0.8457) for E2 and 0.3333 pg/mL (0.2907–0.4065) for E1S, respectively. These LLOQ values were as well as those of previous reported HPLC–RIA and LC–MS/MS.

The precision and accuracy were further examined by analyzing plasma samples spiked with E1 (50 pg/mL), E2 (50 pg/mL) and E1S (300 pg/mL). The concentration of estrogen in human plasma was calculated by subtracting the endogenous estrogen concentration (concentration of blank plasma sample) from the found concentration. The precisions were calculated using found concentrations.

The intra-day ($n = 5$) and inter-day ($n = 3$) assay precisions were within 5.5% and accuracies were within ±5.8% (Table 2).

Finally, this method was used to measure concentrations of E1, E2 and E1S in plasma from healthy women ($n = 5$). We found mean levels of E1, E2 and E1S to be 38.0 pg/mL (range 24.8–53.0), 34.3 pg/mL (22.6–46.6) and 786 pg/mL (163–2080), respectively. As the results, concentration of E1S was higher than those of unconjugated estrogens and showed large individual differences (Table 3).

In conclusion, the use of immunoaffinity extraction could remove the interfering sample matrix that would cause ionization suppression, and markedly improved the sensitivity of LC–MS/MS for estrogens. The method described above has exhibited excellent sensitivity and reproducibility and proved to be applicable for measurement of plasma estrogen levels in healthy women. These results suggested that this method is as useful as the HPLC–RIA in general use. Furthermore, the lower quantitation limits of E1, E2 and E1S would allow determination of changes in estrogen levels in plasma.

References

[1] J.M. Harvey, G.M. Clark, C.K. Osborne, D.C. Allred, J. Clin. Oncol. 17 (1999) 1474.
[2] M.J. van de Vijver, Y.D. He, L.J. van't Veer, H. Dai, A.A.M. Hart, D.W. Voskuil, G.J. Schreiber, J.L. Peterse, C. Roberts, M.J. Marton, M. Parrish, D. Atsma, A. Witteveen, A. Glas, L. Delahaye, T. van der Velde, H. Bartelink, S. Rodenhuis, E.T. Rutgers, S.H. Friend, R. Bernards, N. Engl. J. Med. 347 (2002) 1999.
[3] S.E. Hankinson, W.C. Willet, J.E. Manson, G.A. Colditz, D.J. Hunter, D. Spiegelman, R.L. Barbieri, F.E. Speizer, J. Natl. Cancer. Inst. 90 (1998) 1292.
[4] The Endogenous Hormones and Breast Cancer Collaborative Group, J. Natl. Cancer Inst. 94 (2002) 606.
[5] ATAC Trialists' Group, Lancet 365 (2005) 60.
[6] A.S. Coates, A. Keshaviah, B. Thürlimann, H. Mouridsen, L. Mauriac, J.F. Forbes, R. Paridaens, M.C. Gertsch, R.D. Gelber, M. Colleoni, I. Lang, L.D. Mastro, I. Smith, J. Chirgwin, J.M. Nogaret, T. Pienkowski, A. Wardley, E.H. Jakobsen, K.N. Price, A. Goldhirsch, J. Clin. Oncol. 25 (2007) 486.
[7] R.C. Coombes, E. Hall, L.J. Gibson, R. Paridaens, J. Jassem, T. Delozier, S.E. Jones, I. Alvarez, G. Bertelli, O. Ortmann, A.S. Coats, E. Bajetta, D. Dodwell, R.E. Coleman, L.J. Fallowfield, E. Mickiewicz, J. Andersen, P.E. Lønning, G. Cocconi, A. Stewart, N. Stuart, C.F. Snowdon, M. Carpentieri, G. Massimini, J.M. Bliss, N. Engl. J. Med. 350 (2004) 1081.
[8] J.R. Pasqualini, G. Chetrite, C. Blacker, M.C. Feinstein, L. Delalonde, M. Talbi, C. Maloche, J. Clin. Endocrinol. Metab. 81 (1996) 1460.
[9] J. Geisler, H. Berntsen, P.E. Lønning, J. Steroid Biochem. Mol. Biol. 72 (2000) 259.
[10] A. Martinetti, E. Seregni, E. Bajetta, G.F. Bolelli, L. Ferrari, S. Massaron, C. Botti, E. Bombardieri, Int. J. Biol. Markers 12 (1997) 102.
[11] V. Ingrand, G. Herry, J. Beausse, M.R. de Roubin, J. Chromatogr. A 1020 (2003) 99.
[12] A. Lagana, A. Bacaloni, G. Fago, A. Marino, Rapid Commun. Mass Spectrom. 14 (2000) 401.
[13] R.E. Nelson, S.K. Grebe, D.J. O'Kane, R.J. Singh, Clin. Chem. 50 (2004) 373.
[14] S.S.C. Tai, M.J. Welch, Anal. Chem. 77 (2005) 6359.
[15] K. Yamashita, M. Okuyama, Y. Watanabe, S. Honma, S. Kobayashi, M. Numazawa, Steroids 72 (2007) 819.
[16] S. Ikegawa, K. Matsuura, T. Sato, N.M.R. Isriyanthi, T. Niwa, S. Miyairi, H. Takashina, Y. Kawashima, J. Goto, J. Pharm. Biomed. Anal. 17 (1998) 1.
[17] R. Draisci, L. Palleschi, E. Ferretti, C. Marchiafava, L. Lucentini, P. Cammarata, Analyst 123 (1998) 2605.
[18] J. Yamaguchi, M. Ohmichi, S. Jingu, N. Ogawa, S. Higuchi, Anal. Chem. 71 (1999) 5386.
[19] H. Zhang, J. Henion, Anal. Chem. 71 (1999) 3955.