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Assay for levormeloxifene, a selective estrogen receptor modulator, in human and monkey plasma employing high-performance liquid chromatography and solid-phase extraction

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Abstract

Assays for levormeloxifene, a new selective estrogen receptor modulator, and its 7-desmethyl metabolite in human and cynomolgus monkey plasma are described. Plasma was extracted on mixed-mode bonded sorbent material (C_8/SCX) and the extracts were analysed by high-performance liquid chromatography with fluorescence detection. Recoveries of levormeloxifene and the metabolite exceeded 70%. Within and total assay precision calculated as a coefficient of variation (C.V.) were <8% for both compounds at all concentration levels, except at the limit of quantitation (LOQ) where the C.V. was 15%. Within and total-assay accuracy calculated as a percentage of the nominal value were between 90 and 114% for both analytes. The LOQ was for levormeloxifene and 7-desmethyllevormeloxifene, respectively, 1.5 and 2.5 ng/ml (man) and 5.2 and 6.9 ng/ml (monkey). In the monkey plasma assay, human plasma could substitute monkey plasma as blank plasma.

Keywords: Levormeloxifene

1. Introduction

Estrogen replacement therapy (ERT) is effective in both preventing postmenopausal osteoporosis and reducing the risk of cardiovascular disease [1–2]. However, without the concomitant administration of progesterone supplements, ERT is associated with an increased stimulation of the endometrium causing hyperplasia and risk of cancer [3–5].

Levormeloxifene, ((−)-3,4-*trans*-7-methoxy-2,2-dimethyl-3-phenyl-4-{4-[2-pyrrolidin-1-yl)ethoxy]phenyl}chromane) is a selective estrogen receptor modulator that has been shown to prevent bone loss in the ovariectomised (OVX) rat model of human osteoporosis [6], and to prevent aortic cholesterol accumulation in the OVX rabbit model [7]. In addition, uteri from OVX animals treated with levormeloxifene showed no evidence of epithelial proliferation or glandular stimulation [6,8].

As an alternative to current ERT, levormeloxifene was being developed for the prevention and treatment of postmenopausal osteoporosis. For this medical need clinical development was recently stopped, but new indications are pursued as preclinical testing is being completed.

Levormeloxifene is the l-enantiomer of ormeloxifene and its structure is shown in Fig. 1.

We describe a method for the specific and sensi-

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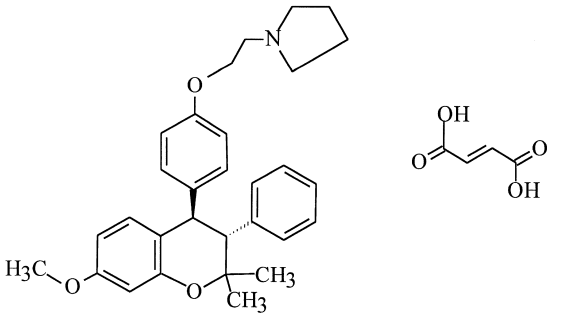

Fig. 1. Chemical structure of levormeloxifene, hydrogen fumarate.

tive determination of levormeloxifene and its major metabolite, 7-desmethyllevormeloxifene, in plasma samples from cynomolgus monkey and man. The analytes are extracted from plasma using a highly selective solid-phase extraction (SPE) technique, and quantified by reversed-phase high-performance liquid chromatography (rHPLC) with fluorescence detection.

For ethical reasons, it would be desirable to minimise the amount of monkey blank plasma. We therefore investigated the possibility of using human plasma as blank plasma in the monkey assay, which would significantly reduce the quantity of monkey plasma required in future studies. The method described has been used for drug assay in several clinical studies [9,10].

2. Experimental

All experimental details apply to both human and monkey plasma assays, unless otherwise stated.

2.1. Chemicals and reagents

Levormeloxifene (hydrogen fumarate), 7-desmethyllevormeloxifene (hydrochloride salt, 0.74 moles crystal water) and the internal standard (I.S.) (a levormeloxifene analogue) were synthesised in-house. Analytical grade acetic acid and *ortho*-phosphoric acid and HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Analytical grade triethylamine (TEA) and ammonium formiate were obtained from Fluka (Buchs, Switzerland), and hydrochloric acid and sodium hydroxide from Bie and Berntsen (Roedovre, Denmark). Demineralised water was purified by passage through a Milli-Q plant from Millipore (Milford, MA, USA). The SPE discs used were C_8/SCX SPEC® PLUS™ Multi-Modal from Ansys (Irvine, CA, USA), containing 30 mg of stationary phase in a reservoir of 3.0 ml. Cynomolgus monkey blank plasma was supplied from Huntingdon Life Science (Huntingdon, UK), and human blank plasma from a local blood donor panel.

2.2. Instrumentation

The HPLC system consisted of a LC Module I from Waters (Milford, MA, USA), a Universal Thermostat Column Heater (Micro-Lab, Hoejbjerg, Denmark) and a 821-FP fluorescence detector (Jasco, Tokyo, Japan). Excitation and emission wavelengths were 279 nm and 305 nm, respectively. Gain was set to ×10, attenuation to 32 and response to standard. The chromatographic system was interfaced to the Expert Ease (version 3.2) data system (Waters, Milford, MA, USA), installed on a Digital Equipment VAX model 4000. Centrifugation of SPE discs was performed using a Megafuge 1.0R (Heraeus Sepatech, Osterrode, Germany), and plasma extracts were evaporated using a TurboVap LV (Zymark, Hopkinton, MA, USA).

Analytical and guard columns were 5 μm particle size Symmetry™ C_{18} columns from Waters (Milford, MA, USA). The dimension of the analytical column was 250 mm×4.6 mm I.D. and the guard column 20 mm×3.9 mm I.D. The pore size of the packing material was 100 Å.

2.3. Buffers for sample preparation

A 0.1 *M* potassium phosphate buffer, pH 3.0 was used to: dilute the samples, equilibrate the SPE discs and together with 1 *M* acetic acid as washing solution. Ammonium formiate buffer 0.1 *M* adjusted

to pH 3.3 with hydrochloric acid was prepared for the mobile phase.

2.4. Preparation of stocks, calibrators and spiked plasma

Stock solutions of 0.1 mg/ml (free base) of levormeloxifene and 7-desmethyllevormeloxifene and of internal standard were prepared in 1*M ortho*-phosphoric acid–methanol (10:90, v/v) and stored at +4°C for maximum 3 months.

Working solutions of the internal standard were prepared in acetonitrile to concentrations of 100 ng/ml and 50 ng/ml.

A pool of plasma calibrators and spiked plasma samples were prepared by adding a small volume of the stock solution to blank plasma. The content of organic solvent was less than 0.9% in the spiked plasma samples. Aliquots of 500 μl were kept frozen at −≤18°C until use.

In the monkey plasma assay, calibrators in plasma from both monkey and man were prepared to contain LOQ-1000 ng/ml of analytes. The calibrators in the human plasma assay covered a range of 1.5–400 ng/ml. In the monkey plasma assay, spiked concentrations for validation were 5.2, 87.0 and 870 ng/ml for levormeloxifene and 6.9, 92.7 and 927 ng/ml for 7-desmethyllevormeloxifene. In the human plasma assay, spiked concentrations for validation were 1.5, 199 and 398 ng/ml for levormeloxifene and 2.5, 202 and 404 ng/ml for 7-desmethyllevormeloxifene.

2.5. Assay procedure

The SPE discs were conditioned with 0.5 ml methanol followed by 0.5 ml phosphate buffer (0.1 *M*, pH 3.0), and centrifuged at 45 *g* for 2 min after each conditioning step.

For the monkey plasma assay, the sample was pretreated by mixing 200 μl of plasma with 500 μl of phosphate buffer and 100 μl of I.S. working solution (100 ng/ml). For the human plasma assay, 500 μl of plasma was mixed with 1 ml of phosphate buffer and 250 μl of internal standard working solution (50 ng/ml). All subsequent steps were identical for both assays.

The pretreated sample was applied directly on top of the conditioned SPE disc and centrifuged at 45 *g* for 3 min, then at 180 *g* for 6 min. The disc was washed consecutively with 0.5 ml acetic acid (1 *M*), 0.5 ml acetonitrile and 0.5 ml phosphate buffer and centrifuged at 180 *g* for 3 min, between the two first steps and at 720 *g* for 5 min following the last step. The SPE discs were eluted with 1 ml of methanol–TEA (98:2, v/v) by centrifugation at 180 *g* for 3 min. The eluates were evaporated to dryness, the residues redissolved in 125 μl of acetonitrile–ammonium formiate buffer (40:60, v/v), and 90 μl injected into the rHPLC system.

The mobile phase consisted of acetonitrile–ammonium formiate buffer (60:40, v/v). Separation was accomplished using changes in flow-rate. The initial flow-rate was 0.7 ml/min for 6 min, followed by 1.3 ml/min for 6 min. The flow was decreased linearly over 1 min to 0.7 ml/min, which was maintained for 2 min before injection of the next sample. The total run time was 15 min. Column temperature was 35°C.

Two series of plasma calibrators were included in each rHPLC run one in the beginning and one in the end. Quantitation was achieved using peak height ratios of analyte and I.S., and calibration curves were generated by the Expert Ease data system using linear regression analysis with weighting of the data points with *1*/concentration.

3. Results

3.1. Chromatographic separation

Representative chromatograms of a standard in reconstitution medium (A), a blank monkey or human plasma extract (B), an extract of monkey or human plasma spiked with levormeloxifene, 7-desmethyllevormeloxifene and internal standard (C) and an extract of a plasma sample obtained from a postmenopausal volunteer 2 h after a single oral dose of 30 mg of levormeloxifene (D) are shown in Figs. 2 and 3, respectively. As seen in Figs. 2A and 3A levormeloxifene, 7-desmethyllevormeloxifene and internal standard with retention times of 5 min, 7.5 min and 10 min, respectively, were well separated.

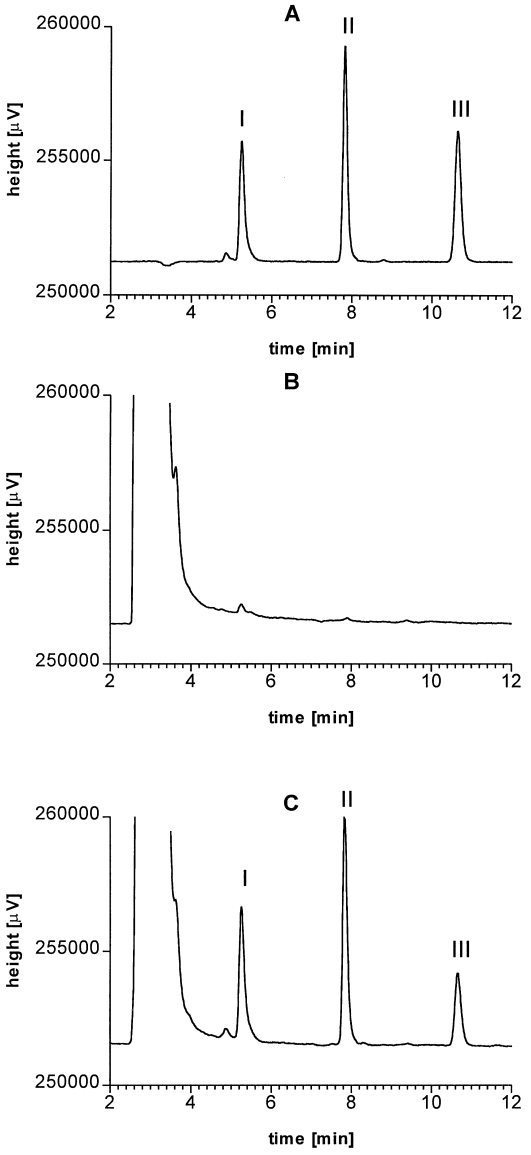

Fig. 2. Sample chromatograms for (A) standard in reconstitution medium containing 100 ng/ml of analytes and I.S., (B) blank monkey plasma, and (C) monkey plasma spiked with 100 ng/ml of levormeloxifene and 7-desmethyllevormeloxifene after 1.6 fold concentration.

In plasma from a volunteer dosed orally with levormeloxifene separation of the analytes and I.S. was likewise adequate (Fig. 3D).

3.2. Linearity

The calibration curves in both assays proved to be linear and reproducible for levormeloxifene and 7-desmethyllevormeloxifene over the concentration range tested (Table 1).

For the monkey plasma assay, the slope and intercept of the calibration curves prepared in monkey or human plasma did not differ significantly (Student's *t*-test, $p>0.05$). It is therefore valid to use human plasma as matrix for the preparation of calibrators when assaying monkey samples.

3.3. Precision, accuracy, limit of quantitation and recovery.

Estimation of within and total (within and between) run accuracy and precision were accomplished by assaying six replicates of spiked samples at three concentration levels (low, medium and high) in one assay series and one sample at each level in six assay series, respectively. The limit of quantitation (LOQ) was defined as the lowest concentration in plasma yielding a within assay variation below 20% and an accuracy between 80 and 120%.

The LOQ were estimated to be 1.5 ng/ml and 2.5 ng/ml for levormeloxifene and 7-desmethyllevormeloxifene, respectively in the human plasma assay (Tables 2 and 3). Irrespective of the matrix used for preparation of plasma calibrators, the LOQ values in the monkey plasma assay were 5.2 ng/ml and 6.9 ng/ml for levormeloxifene and 7-desmethyllevormeloxifene, respectively.

The within assay and total assay run precision were $<8\%$ for both compounds and assays at all concentration levels except at the LOQ, where total run results varied up to 12 and 15% for 7-desmethyllevormeloxifene in the human and monkey plasma assay, respectively. Within and total run accuracy were satisfactory as no value for accuracy was below 90% or exceeded 115% (Tables 2 and 3).

Recoveries of levormeloxifene and 7-desmethyllevormeloxifene were calculated from the ratio of the slopes of peak heights versus concentration obtained from plasma samples and from non extracted reference solutions. Recovery of the internal standard was calculated from the ratio of peak heights.

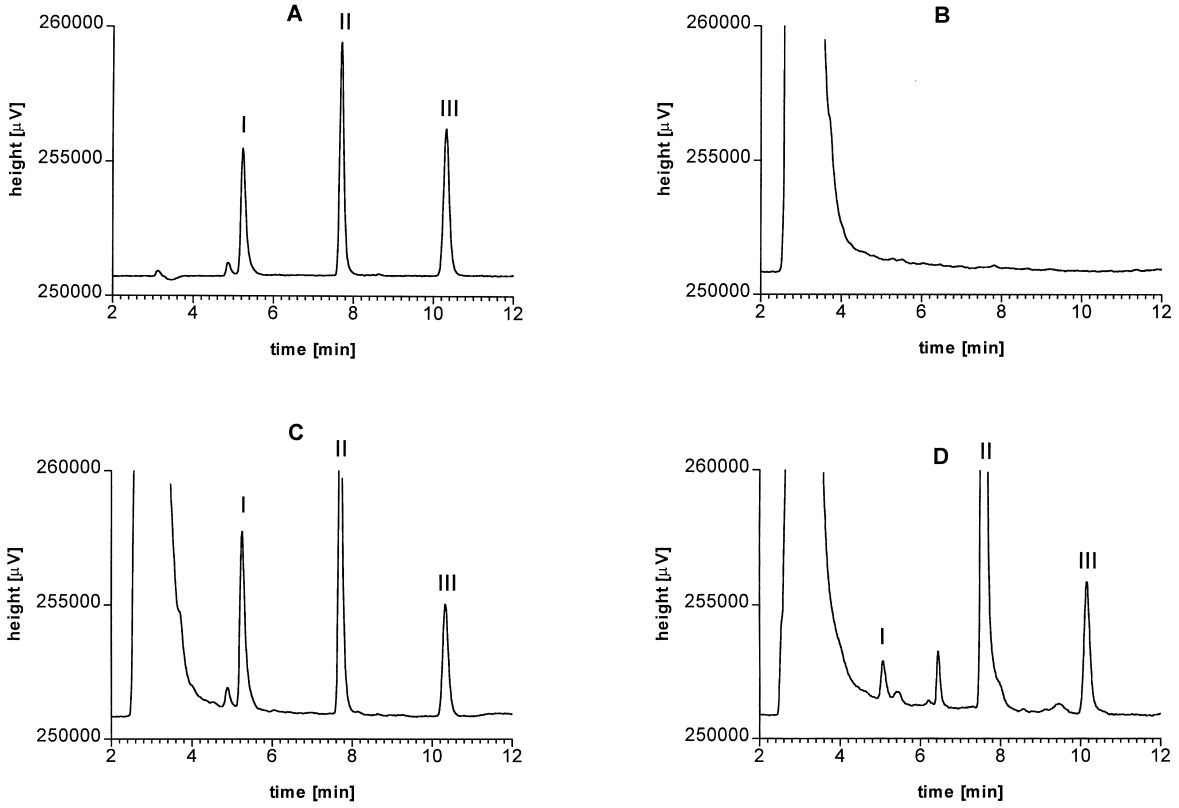

Fig. 3. Sample chromatograms for (A) standard in reconstitution medium containing 100 ng/ml of analytes and I.S., (B) blank human plasma, (C) human plasma spiked with 50 ng/ml of levormeloxifene and 7-desmethyllevormeloxifene after 3.2 fold concentration, and (D) plasma from a healthy postmenopausal woman dosed with 30 mg of a solution of levormeloxifene-2 h post dosing (concentration of levormeloxifene and 7-desmethyllevormeloxifene were found to be 132.5 ng/ml and 7.5 ng/ml, respectively). Peak names: I: 7-desmethyllevormeloxifene; II: levormeloxifene; III: Internal standard.

Table 1
Assay linearity. All values are calculated from six calibration curves, each comprising of at least 12 data points

Plasma type and analyte	Slope Mean±SD $\times 10^{-2}$	Intercept Mean±SD $\times 10^{-2}$	r^2 Mean±SD
Human plasma			
Levormeloxifene	6.615±0.358	−0.736±0.460	0.997±0.002
7-Desmethyllevormeloxifene	3.139±0.196	0.299±0.562	0.994±0.004
Monkey plasma			
Levormeloxifene[a]	3.451±0.153	11.17±23.65	0.999±0.001
Levormeloxifene[b]	3.399±0.151	11.42±25.66	0.998±0.001
7-Desmethyllevormeloxifene[a]	1.615±0.084	5.436±6.023	0.999±0.001
7-Desmethyllevormeloxifene[b]	1.569±0.063	2.832±5.855	0.997±0.001

[a] Calibration standards prepared in monkey plasma.
[b] Calibration standards prepared in human plasma.

Table 2
Accuracy and precision within and between runs for levormeloxifene[a]

Plasma type	Nominal concentration (ng/ml)	Within-run			Total (within and between runs)		
		Concentration found Mean±SD (ng/ml)	Accuracy (%)	Precision (C.V.%)	Concentration found Mean±SD (ng/ml)	Accuracy (%)	Precision (C.V.%)
Human	1.5	1.6±0.1	107	4.0	1.5±0.1	103	5.4
	199	204±10.7	103	5.2	189±6.7	95	3.5
	398	405±4.5	102	3.6	379±12.4	95	3.3
Monkey[b]	5.2	6.0±0.4	114	6.6	4.7±0.4	90	9.4
	87.0	84.9±2.9	98	3.4	78.0±1.5	90	1.9
	870	884±15.3	102	1.7	815±28.7	94	3.5
Monkey[c]	5.2	5.4±0.4	103	7.4	4.7±0.3	90	6.6
	87.0	84.8±2.9	97	3.4	79.2±2.4	91	3.0
	870	889±15.4	102	1.7	827±32.8	95	4.0

[a] The calculations of mean, SD, accuracy and precision were performed before rounding. The numbers presented in Tables 2 and 3 are rounded numbers.
[b] Calibration standards prepared in monkey plasma.
[c] Calibration standards prepared in human plasma.

The recovery exceeded 70% in both assays except for the I.S., where the recovery was 65% in the human plasma assay (Table 4).

3.4. Selectivity

The selectivity of the monkey and the human plasma assay was investigated by assaying blank plasma from six independent sources/volunteers and inspecting the chromatograms for endogenous impurities, which could possibly interfere with the measurement of analytes and/or I.S. Interfering peaks were not present in the chromatograms at the retention times of levormeloxifene, 7-desmethyllevormeloxifene and internal standard (Figs. 2B and 3B).

Table 3
Accuracy and precision within and between runs for 7-desmethyllevormeloxifene[a]

Plasma type	Nominal concentration (ng/ml)	Within-run			Total (within and between runs)		
		Concentration found Mean±SD (ng/ml)	Accuracy (%)	Precision (C.V.%)	Concentration found Mean±SD (ng/ml)	Accuracy (%)	Precision (C.V.%)
Human	2.5	2.7±0.1	107	4.8	2.8±0.3	111	11.9
	202	206±12.3	102	6.0	195±8.4	97	4.3
	404	413±19.8	102	4.8	386±21.0	96	5.4
Monkey[b]	6.9	7.2±0.3	103	4.1	6.7±1.0	96	15.0
	92.7	92.3±3.4	100	3.6	85.5±4.4	92	5.2
	927	977±22.7	105	2.3	899±27.0	97	3.0
Monkey[c]	6.9	7.4±0.3	106	4.0	8.0±1.2	114	15.0
	92.7	92.3±3.4	100	3.6	87.1±6.4	94	7.4
	927	975±22.6	105	2.3	928±23.7	100	2.6

[a] The calculations of mean, SD, accuracy and precision were performed before rounding. The numbers presented in Tables 2 and 3 are rounded numbers.
[b] Calibration standards prepared in monkey plasma.
[c] Calibration standards prepared in human plasma.

Table 4
Recovery of levormeloxifene, 7-desmethyllevormeloxifene and internal standard from human and monkey plasma

Analyte	Recovery, %	
	Human plasma	Monkey plasma
Levormeloxifene	75	73
7-Desmethyllevormeloxifene	73	70
Internal standard	65	81

The 2.2-didesmethyl metabolite of levormeloxifene, which has been identified in human plasma, did not elute with any of the analytes (retention time: 6.2 min).

3.5. Stability of plasma samples

The long-term stability of levormeloxifene and 7-desmethyllevormeloxifene in plasma was investigated by storing spiked samples at three different concentration levels (10, 200 and 400 ng/ml-human plasma; 20, 100 and 1000 ng/ml-monkey plasma) in portions of 1.2 ml at ≤ -18°C for 11 months.

Neither in human nor in monkey plasma signs of deterioration were observed during this time span (results not shown).

4. Discussion

Solid phase extraction is a highly efficient sample preparation technique for the clean up of biological samples [11]. A wide range of SPE columns and discs with different sorbent properties are available. Despite this the extraction of non-polar compounds from plasma is mostly performed on sorbent material containing modified reversed-phase silicas such as C_{18} and C_8 [12]. However for a number of basic and non-polar compounds sample clean-up using mixed-mode bonded phases has been reported. The concept of mixed-mode extraction implies combining different mechanism of retention, usually hydrophobic interaction and ion-exchange. Isolation of a substantial number of drugs from biological matrices among those: methadone, codeine, morphine, cocaine, flunitrazepam, clenbuterol and amphetamine has successfully been accomplished using mixed-mode bonded silicas [13–19].

As levormeloxifene and its major metabolite are bases (pK_a: 9.6), highly lipophilic and contain an ionizable pyrrolidine group we decided, with reference to [13–19], to take advantage of the dual nature of the molecules and to develop a SPE method on a mixed-mode stationary phase (C_8/SCX) followed by chromatography on a different phase (C_{18}). For the clean-up we preferred discs to cartridges as smaller volumes of solvent usually are required and less channelling effects using discs have been reported [18]. On the C_8/SCX support the silica bounded octyl groups are primarily responsible for non-polar interaction, whereas the benzenesulfonyl groups (SCX) have both non-polar and strong cation-exchange properties [20].

When plasma samples containing levormeloxifene and 7-desmethyllevormeloxifene are mixed with phosphate buffer adjusted to pH 3.0 and loaded on the discs, we propose that the analytes become charged and are retained by both hydrophobic and ionic mechanisms. Because the analytes are retained by two mechanisms and ion-exchange interaction is of higher energy than non-polar interactions it is possible to remove many biological interferences primarily retained by non-specific hydrophobic interaction by a solvent rinse without compromising isolates retention.

In this case washing with acetic acid and pure acetonitrile serve to remove acid-soluble and primary non-polar interferences from the discs so that only the analytes and possibly basic endogenous substances with ionic interaction are retained. The analytes are finally eluted from the discs with methanol–TEA (98:2, v/v), that disrupts both the ionic and the non-polar interactions simultaneously.

The wash procedure was evaluated to clarify how much and what proportion of organic solvent was necessary to remove as many interferences as possible without introducing significant breakthrough of analytes. This was a critical step as less than 0.5 ml pure acetonitrile improved the recovery of the analytes somewhat, but the extracts were then more impure. With the washing step used, very clean plasma extracts were obtained and the recovery of the analytes was sufficiently high and constant to get reproducible results. Thus, reconstitution in small volumes was possible to obtain high assay sensitivity.

In the early experiments of assay development, when samples were diluted only with buffer, recoveries of the analytes were poor in general: less than 50%. As the analytes are highly protein-bound [21] the poor recovery was thought to be a result of insufficient retention on the extraction columns. The recovery improved considerably when acetonitrile up to 10–15% of the total sample volume was mixed with the plasma sample, before loading to the disc.

In conclusion, the sensitivity, selectivity, level of accuracy and precision described here were found to be well suited for assay of levormeloxifene and its major metabolite in plasma from man and monkey. In the monkey plasma assay, it was shown that the calibration curve did not differ significantly if human plasma was used for the calibration samples rather than monkey plasma. Thus, it is valid to use human plasma as matrix for calibrators, when assaying levormeloxifene and 7-desmethyllevormeloxifene in plasma from cynomolgus monkeys. The method has successfully been used in several clinical studies [9–10] as well as for the analysis of samples from safety and efficacy studies in cynomolgus monkeys (not published).

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