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Validation of an LC-MS assay for the quantification of the enantiomers of Org 4428 in human plasma[1]

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Abstract

A sensitive and selective liquid chromatographic mass spectrometric assay has been validated for the quantification of Org 4428 enantiomers in human plasma. The assay employs *n*-hexane extraction from alkalinized plasma, separation on a narrow-bore enantioselective normal phase Chiralpak AD column and APCI MS-MS detection. The lower limit of quantification is 0.5 ng ml^{-1} plasma for the individual enantiomers. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

In in vitro model systems Org 4428 (±) *cis*-1,3,4,13b-tetrahydro-2,10-dimethyldibenz[2,3:6,7] oxepino[4,5-*c*]pyridin-4a(2H)-ol (Fig. 1) is a potent and specific inhibitor of synaptosomal noradrenalin reuptake. Org 4428 does not exhibit affinity for adrenergic, histaminergic, muscarinic or dopaminergic receptors and only weak affinity for 5-HT_2 and 5-HT_D receptors. As such, Org 4428 qualified to be a candidate for the treatment

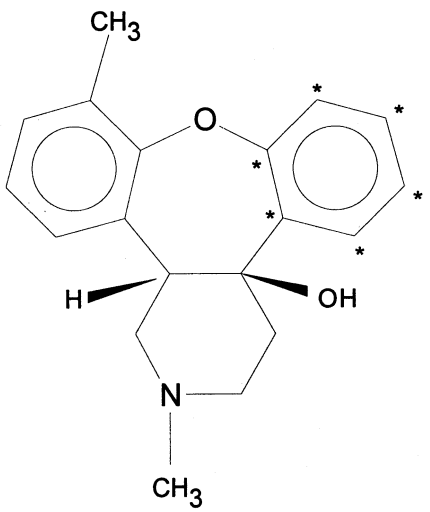

Fig. 1. Molecular structure of Org 4428. The six ^{13}C labelling sites (internal standard) are indicated with asterisks.

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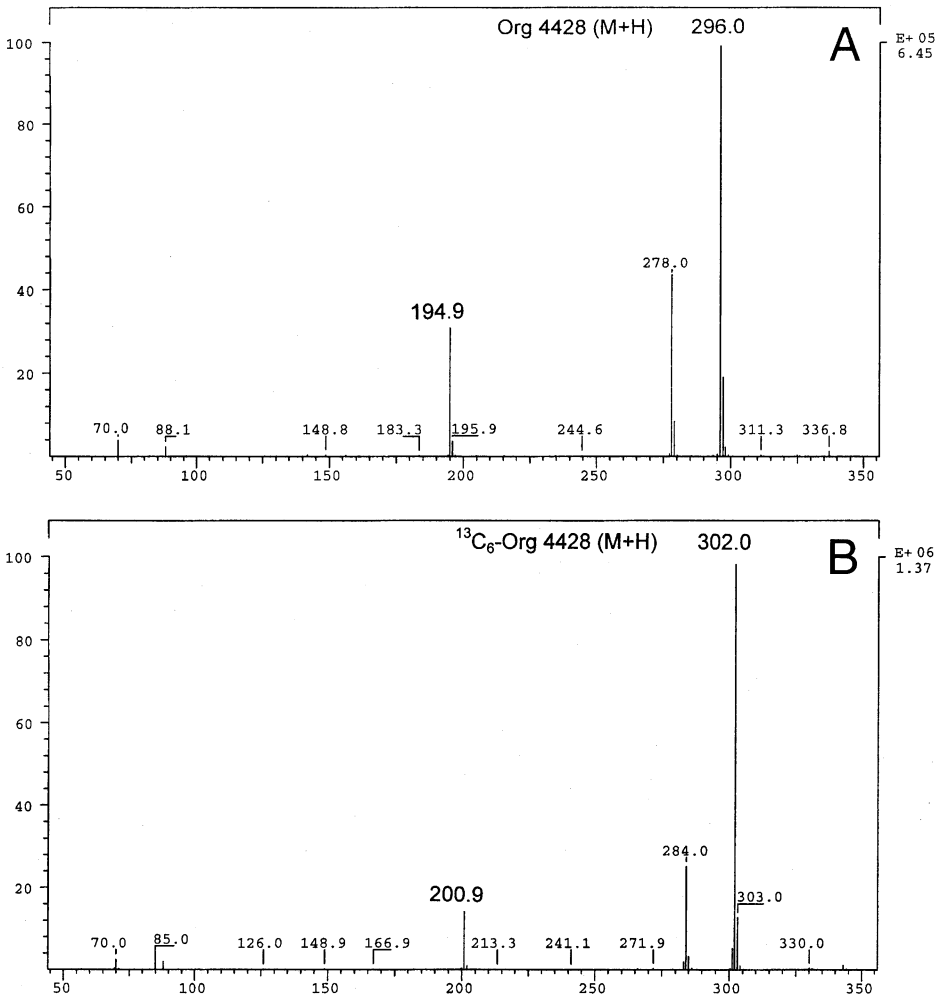

Fig. 2. Mass spectra of Org 4428 (A) and $^{13}C_6$-Org 4428 (B). MS conditions: vaporizer, 240°C; capillary, 140°C; corona needle, 6.0 μA.

of major depression. Phase I clinical trials with total single daily doses up to 1000 mg day^{-1} and 2×400 mg divided doses for 11 days did not reveal intolerable side effects. Pharmacokinetics was found to be linear over the dose range that was investigated. The elimination half life was found to be approximately 11–14 h. In subsequent clinical trials, Org 4428 has shown insufficient efficacy for the treatment of major depression. Consequently, further development of this indication was stopped.

The pharmacokinetics of the individual enantiomers of Org 4428 was to be studied upon administration of the (racemic) Org 4428 to man. To that end an assay method had to be developed and validated.

Paanakker et al. have described the extraction of mirtazapine (Remeron®), an antidepressant with a comparable structure, from alkalinized plasma [1]. This mode of extraction was used for the extraction of (+) Org 4428 and (−) Org 4428 from plasma. Since the available racemic

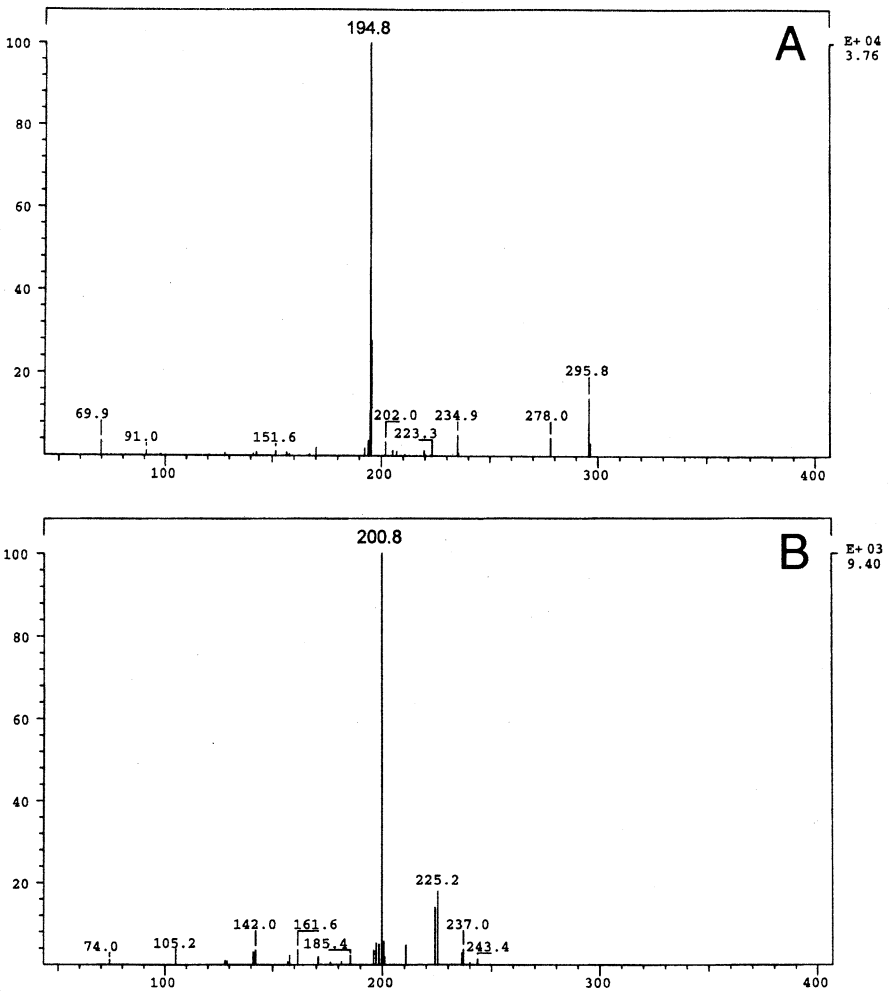

Fig. 3. Mass spectra of the product ions of Org 4428 (A) and $^{13}C_6$-Org 4428 (B). MS–MS conditions, see Fig. 2; collision gas, argon.

assay successfully employs gas chromatography (GC) with nitrogen selective detection, at first it was tried to use enantioselective GC. Although a satisfactory separation could be achieved on a derivatized β-cyclodextrin column (Chiraldex β PH), run times amounted up to 45 min and selectivity remained a problem. The availability of LC-MS and new types of enantioselective phases enabled the switch from GC to LC. The versatility of LC-MS was already demonstrated for the pharmaceutical analysis of Org 4428 by Debets et al. [2].

It was investigated whether $^{13}C_6$-Org 4428 (Fig. 1) could be used as the internal standard (IS): $^{13}C_6$-Org 4428 contains equimolar amounts of (+) and (−) $^{13}C_6$-Org 4428 which each could serve as IS for (+) and (−) $^{12}C_6$-Org 4428, respectively. No suitable reversed-phase systems using enantioselective columns could be found for baseline separation of the individual enantiomers.

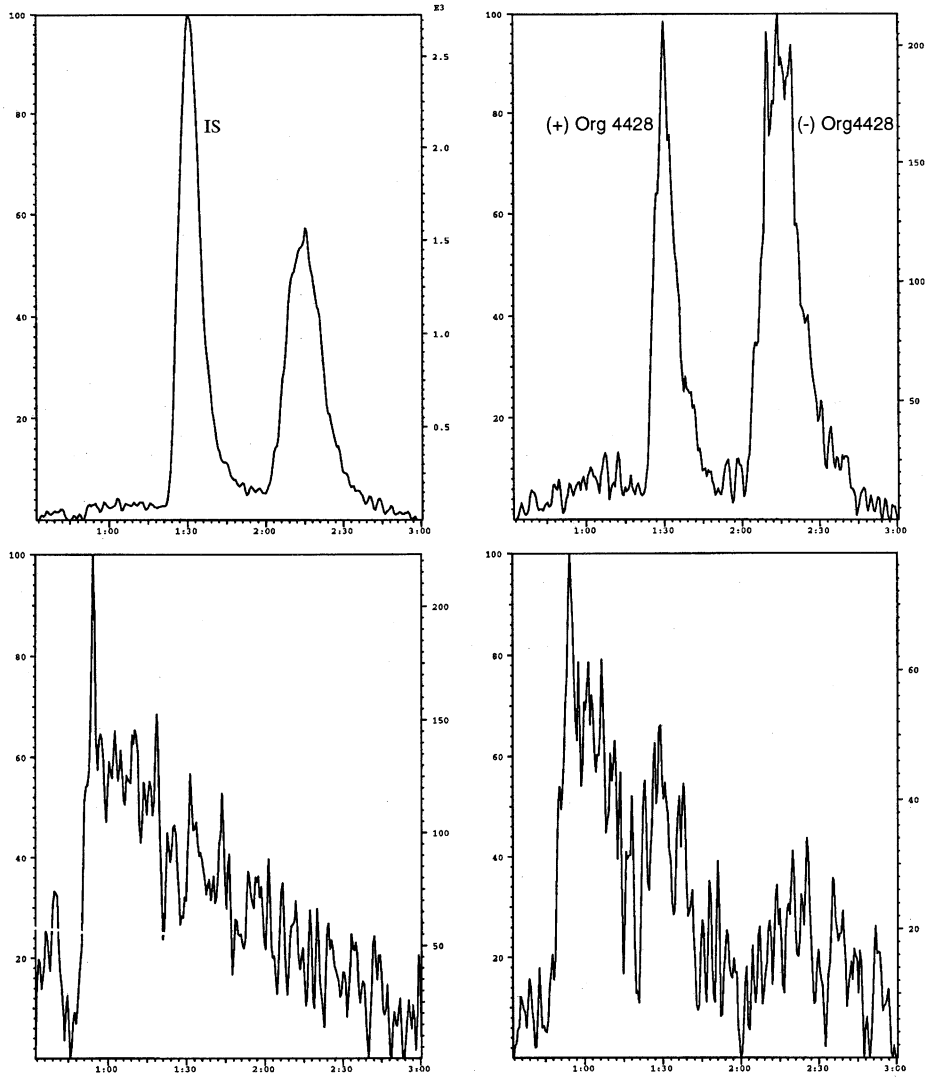

Fig. 4. Chromatograms of (bottom) a blank human plasma extract and (top) an extract of plasma spiked with 0.5 ng (+) Org 4428/(−) Org 4428 and 20 ng (racemic) $^{13}C_6$-Org 4428. Mass traces for the IS (m/z 201) and analyte (m/z 195) are depicted on the left and right, respectively. LC conditions were: Chiralpak AD, 100 × 1 mm (I.D.); mobile phase, hexane/methanol/ethanol (95:3:2; v/v/v); inj. volume, 5 μl; flowrate, 0.125 ml min^{-1}; for MS–MS conditions, see Fig. 3.

Therefore, a straight-phase system was used. It turned out that (+) $^{13}C_6$-Org 4428 had to be used as IS for both (+) and (−) $^{12}C_6$-Org 4428, since the extract of blank plasma showed an interfering chromatographic peak adjacent to (−)$^{13}C_6$-Org 4428 obstructing an accurate quantification.

For assay validation the 'Arlington' guidelines were adhered to [3].

2. Materials and methods

2.1. Reagents

Methanol (Uvasol), *n*-hexane (Uvasol) and ammonia solution (Suprapur, 25%) were purchased from Merck, Darmstadt, Germany. Ethanol (p.a.

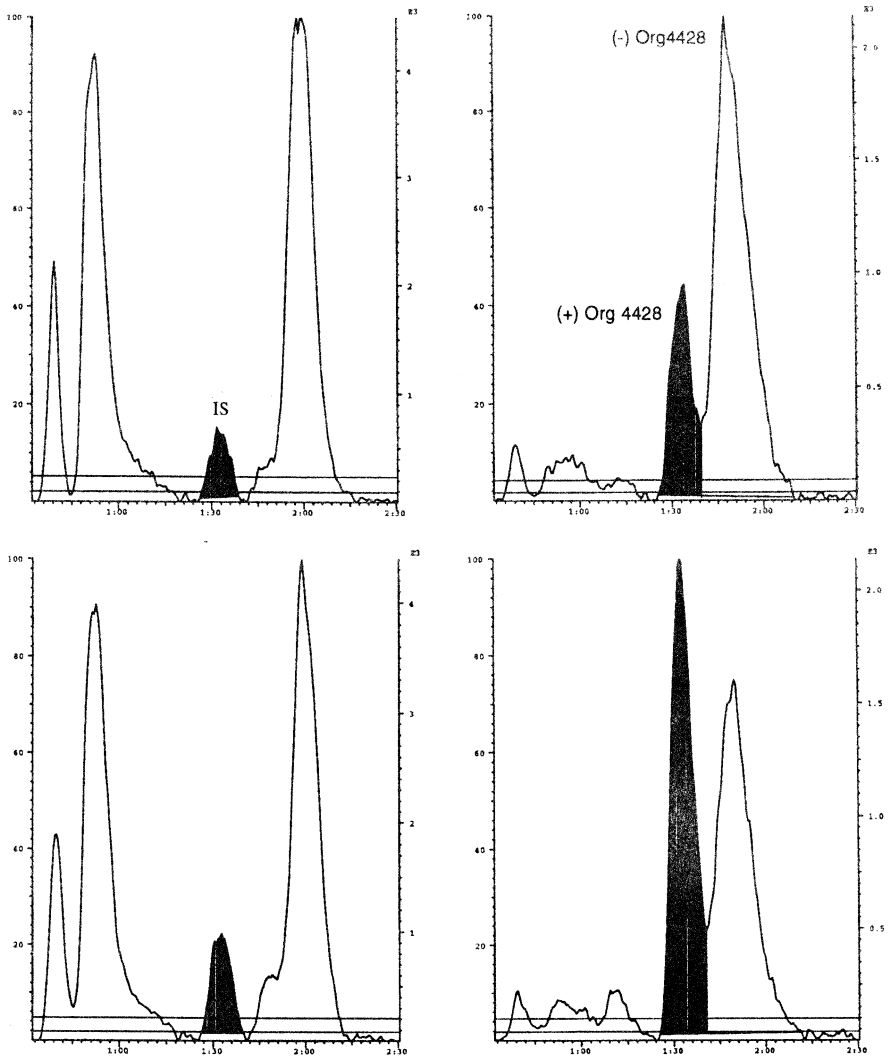

Fig. 5. Chromatograms of extracts from human plasma, obtained at 1 (top) and 4 h (bottom) after a single 25 mg dose of Org 4428. For LC-MS conditions, see Fig. 4.

quality) was obtained from Diosynth, Oss, Netherlands.

2.2. Extraction procedure

All plasma samples (1.0 ml) were spiked with 20 ng IS. After 15 min equilibration, 100 μl 25% aqueous ammonia was added. After mixing thoroughly, extraction was performed with 8 ml *n*-hexane by whirl-mixing. After 4 min centrifugation for phase separation, the *n*-hexane layer was transferred for evaporation-to-dryness. The residue was dissolved in 200 μl ethanol, re-evaporated and finally dissolved in 5 μl LC solvent, which was auto injected onto the LC-MS.

2.3. Apparatus

A Hewlett Packard 1050 system (Amstelveen, Netherlands) was used equipped with an autosam-

Table 1
Calibration curve equations using 1/(concentration)^2 weighting

Analyte	Slope	Intercept	correlation coefficient (r)
(+) Org 4428	0.0996 ± 0.0063	0.0192 ± 0.0113	0.9936 range: 0.9916–0.9959
(−) Org 4428	0.0852 ± 0.0059	0.0108 ± 0.0124	0.9933 range: 0.9905–0.9968

Values are expressed as mean ± S.D. averaged over all runs.

pler. A narrow-bore Chiralpak AD (Daicel Chemical Industries) column was used (100×1 mm I.D., particle size 10 μm, custom-packed by LC Packings, Amsterdam, Netherlands). The mobile phase consisted of *n*-hexane/methanol/ethanol (95:3:2 v/v/v) and was delivered at 0.125 ml min^{-1}. For detection a Finnigan-MAT TSQ-7000 (Bremen, Germany) mass spectrometer was used equipped with an Atmospheric Pressure Chemical Ionization (APCI) source. The vaporizer was set at 240°C and the capillary at 140°C, the corona needle was operated at 6.0 μA. The sheath gas was adjusted to 30 psi. Argon was used as collision gas. The mass spectrometer was operated under MS-MS conditions, monitoring product ions of $^{12}C_6$-Org 4428 at m/z 195 and at m/z 201 for $^{13}C_6$-Org 4428.

2.4. Linearity and calibration

The linearity of the assay was evaluated by duplicate analysis of eight plasma calibration standards containing 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng ml^{-1} (+) and (−) Org 4428 with a fixed amount of 20 ng IS ($^{13}C_6$-Org 4428). In addition, blank human plasma was spiked with 20 ng ml^{-1} IS to quantify a possible endogenous interference. This was repeated in six separate calibration series. Calibration curves were constructed using 1/(concentration)^2 weighted linear regression (peak height ratio of the analyte of interest versus the IS, plotted against the concentration.

2.5. Sensitivity

The lower limits of quantification (LOQ) were assessed for (+) and (−) Org 4428 during the six validation series. The LOQ is defined as that concentration of the calibration curve that can be determined in six validation series with an accuracy (expressed as % nominal concentration) between 80 and 120% and a precision (coefficient of variation) below 20%.

2.6. Precision and accuracy

Precision (CV%) and accuracy (% nominal) were determined in six separate runs by replicate analysis of three quality control plasma pools at (+) and (−) Org 4428 concentrations of 2, 10 and 75 ng ml^{-1} plasma. Of the QC pools, 1.0 ml plasma containing 2, 10 and 75 ng ml^{-1} (+) and (−) Org 4428 was processed in triplicate. A fixed amount of 20 ng IS was added. The QC plasma pools were stored at T = −20°C.

Inter-assay precision (CV%) and accuracy (% nominal) were assessed for each QC pool by triplicate analysis in five separate runs and six-fold analysis in one run.

Intra-assay precision (CV%) and accuracy (% nominal) were assessed for each QC pool by six-fold analysis of the QC pools in one run.

2.7. Freeze/thaw cycles

The influence of frequent freezing and thawing was investigated by comparing the results of the three QC pools after one freeze/thaw cycle, with those of two and three times freezing and thawing.

2.8. Racemization

In order to study possible racemization vice versa, (+) and (−) Org 4428 were processed separately according to the assay procedure at the 75 ng ml^{-1} plasma level in triplicate. These samples were analyzed directly and after storage

Table 2
Accuracy and precision of calibration samples over six validation series[a]

Analyte		Nominal concentration (ng ml^{-1})							
		0.5	1	2.5	5	10	25	50	100
(+) Org 4428	Acc.[b]	96.2	106.3	99.1	103.5	104.1	100.8	97.1	93.0
	Prec.[c]	16.8	10.6	9.0	6.2	5.8	3.7	7.7	3.4
	n	12	13	12	13	14	14	14	14
(−) Org 4428	Acc.[b]	96.6	106.0	98.5	104.0	104.6	101.6	97.3	91.9
	Prec.[c]	12.5	9.3	8.2	8.6	5.7	8.7	9.7	5. 9
	n	12	12	11	13	14	14	14	14

[a] Calculated using 1/(concentration)^2 weighted linear regression.
[b] Accuracy, percentage of the nominal concentration.
[c] Precision, coefficient of variation.

overnight, in the auto-sampler tray, at ambient temperature.

2.9. Recovery

The extraction recovery of (+) and (−) Org 4428 from human plasma was assessed by processing the 2, 10 and 75 ng ml^{-1} QCs ($n = 6$) according to the assay procedure without adding IS. Prior to injection onto LC-MS a fixed amount of 20 ng IS was added to the extracted residue of each QC sample. Standards ($n = 3$ at each concentration) containing 2, 10 and 75 ng of (+) and (−) Org 4428 and a fixed amount of 20 ng IS (without further processing) were also injected onto LC-MS.

The recovery of (+) and (−) Org 4428 was calculated by comparing the signal ratios of each enantiomer and its IS, in the individual extracted plasma samples, with the mean of signal ratios of (+) and (−) Org 4428 standards and their IS of the diluted standards.

2.10. Selectivity

Blank human plasma from six different donors as well as blank water were analyzed according to the assay procedure. In addition, diluted standards of (+) and (−) Org 4428 and their internal standards were analyzed separately to assess whether (+) and (−) Org 4428 interfere with each other or with their IS and vice versa.

3. Results and discussion

3.1. Chromatography

Among the various types of enantioselective stationary phases currently available, polysaccharide based materials play an important role [4–6]. Of the columns tested the 3,5 dimethylcarbamate derivatized amylose based Chiralpak AD [6] was the only to produce a good resolution. The results with this column for several structural analogues clearly showed the improvement of column materials as compared with a survey from some years ago [7].

In order to improve selectivity, it appeared necessary to use tandem mass spectrometry. Fig. 2 shows the mass spectra of racemic Org 4428 and its ^{13}C substituted IS obtained with single MS and Fig. 3 shows the respective product ion mass spectra. Under optimal MS-MS conditions, only one important product ion was formed, which indeed contained the isotopically labelled ring system. Thus, this ion was chosen for quantitative monitoring.

Using the tandem approach, chromatographic run times of less than 3 min could be achieved without interfering peaks from plasma constituents. Of course, this is only allowed when the two enantiomers are baseline separated in this time frame. This could be achieved by using a ternary mobile phase with both methanol and ethanol as modifiers. Sensitivity was improved by

Table 3
Intra- and inter assay accuracy and precision

Nominal concentration (ng ml^{-1})	Intra-day				Inter-day			
	(+) Org 4428		(−) Org 4428		(+) Org 4428		(−) Org 4428	
	Acc.[a]	Prec.[b]	Acc.	Prec.	Acc.	Prec.	Acc.	Prec.
2	106.6	2.8	94.7	4.5	107.6	10.2	97.8	13.3
10	104.1	5.3	100.8	9.1	107.2	4.3	102.7	8.0
75	91.1	2.1	92.0	2.4	95.3	5.5	89.9	8.6
n	5–6		5–6		29–33		29–33	

[a] Accuracy, percentage of the nominal concentration.
[b] Precision, coefficient of variation.

using a narrow-bore column instead of the commercially available 50 × 4.6 mm I.D. Another advantage of the narrow-bore column is the reduction of waste through flow reduction. Chromatograms of plasma extracts, obtained from both blank plasma and plasma spiked at the lowest calibration level (0.5 ng ml^{-1} of both enantiomers) are depicted in Fig. 4. No interferences in the blank plasma traces are seen (note the different right *Y*-axis as compared with the spiked plasma extract), which was the case for all six donor plasmas tested. Applicability to clinical samples is demonstrated in Fig. 5, which shows chromatograms of plasma extracts prepared from samples obtained at 1 and 4 h after single oral administration of 25 mg. Because these samples were run before the actual validation, retention times and chromatographic conditions differ slightly from those in Fig. 3. It can be seen from the chromatograms that (−) Org 4428 appears in the blood faster than its enantiomer, but it is also cleared faster. This implies that (+) Org 4428/(−) Org 4428 ratios can be both below and above one, ranging from approximately 0.1–10. Since the development for major depression was cancelled shortly before the assay could be applied, no other samples became available for the assay.

Table 4
Recovery

Concentration (ng ml^{-1})	Recovery (%)	
	(+) Org 4428	(−) Org 4428
2	61.1 ± 7.7	50.2 ± 6.8
10	60.5 ± 4.7	54.0 ± 5.8
75	62.9 ± 2.5	56.0 ± 2.6
n	6	6

Values are expressed as percentage of the nominal concentration ± S.D.

3.2. Validation

Results of the assay performance are summarized in Tables 1–5. Table 1 shows the calibration curve equations and correlation coefficients, as determined using linear regression, with a weighting factor of 1/(concentration)^2.

Table 2 shows the mean back-calculated concentrations of the calibration standards over six validation series. The lower limit of quantification for both enantiomers was set at 0.5 ng ml^{-1}, the precision at this concentration being better than 20% and the accuracy being between 80 and 120% of the nominal concentration.

Table 3 shows the data obtained with intra- and inter-day accuracy and repeatability using quality control samples at three representative levels. Intra-day accuracy was between 91.1 and 106.6% of

Table 5
Freeze/thaw stability

Concentration (ng ml^{-1})	Freeze/thaw stability			
	(+) Org 4428		(−) Org 4428	
	1 cycle	3 cycles	1 cycle	3 cycles
2	125.4 ± 18.6	100.1 ± 2.2	111.5 ± 14.6	82.0 ± 5.3
10	109.9 ± 3.4	108.5 ± 4.0	102.4 ± 11.9	97.1 ± 8.0
75	97.7 ± 2.7	100.7 ± 1.4	90.3 ± 8.7	91.5 ± 5.3
n	3	3	3	3

Values are expressed as percentage of the nominal concentration ± S.D.

the nominal concentration of (+) Org 4428, with figures for (−) Org 4428 being slightly better. Inter-day accuracy ranged from 95.3 to 107.6% for (+) Org 4428, and from 89.9 to 102.7% for (−) Org 4428. Precision was better than 15% in all cases. Results on recovery are given in Table 4. Surprisingly, recovery of both enantiomers was not exactly identical. No concentration dependency was seen. The stability results shown in Table 5 confirm that both enantiomers are stable upon repeated freezing and thawing: no systematic trend was observed. For clarity, results of two times freezing and thawing have not been tabulated. To exclude the possibility of racemization, plasma extracts containing only one of both enantiomers were prepared and reinjected after standing for 24 h at room temperature. No formation of the other enantiomer was observed (results not shown).

In conclusion, a fast and sensitive assay to study enantiomer pharmacokinetics of Org 4428 has been developed and validated.

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