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On-line turbulent-flow chromatography–high-performance liquid chromatography–mass spectrometry for fast sample preparation and quantitation

Lars Ynddal [a,*], Steen Honoré Hansen [b]

[a] *Department of Drug Metabolism, Research and Development, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Maaloev, Denmark*
[b] *Department of Analytical Chemistry, The Danish University of Pharmaceutical Sciences, DK-2100 Copenhagen, Denmark*

Abstract

A sensitive and selective liquid chromatography–mass spectrometry method has been developed for the simultaneous identification and quantitation of drug substances and metabolites in rat plasma. The method combines on-line turbulent-flow chromatography, high-performance liquid chromatography and mass spectrometry. This combination is considered to be a new approach suitable for fast bio-analysis in drug discovery. Dextromethorphan, and its two metabolites, dextrorphan and 3-methoxymorphinan served as model substances. The analytes present in plasma were collected on a Cyclone column using turbulent-flow chromatography and were subsequently transferred on-line to and focused on an X-Terra MS C_8 column. The analytes were eluted by a linear gradient and detected by a fast scanning mass spectrometer. The detector response was quadratic and the dynamic range was estimated to be 0.5–100 ng/ml plasma or 12.5 pg to 2.50 ng injected into the system.

Keywords: Turbulent flow chromatography; Sample preparation; Dextromethorphan; Dextrorphan; 3-Methoxymorphinan

1. Introduction

Turbulent-flow chromatography (TFC) was introduced by Quinn and Takarewski [1] and has been established for quantitative determination of drugs in biological matrices [2]. TFC is used both in single-column mode [3] or connected on-line to high-performance liquid chromatography (HPLC) column for subsequent separation prior to the introduction to the detector [4]. The detector is in most cases a mass spectrometer.

Sample preparation using TFC is fast and easy and much less labour intensive compared to the traditional off-line sample preparations such as liquid–liquid or solid-phase extractions [2,3].

TFC combined with HPLC has been applied for the investigation of microsomal stability and for in vitro metabolite profiling [5]. The integration of quantitative and qualitative analysis for in vivo ADME (absorption, distribution, metabolism and excretion) drug discovery support has been reported by Poon et al. [6] and Zhang et al. [7] but they both used off-line, non-turbulent sample preparation techniques. The use of TFC for sample clean-up followed by HPLC separation and coupled to a fast scanning

* Corresponding author.
E-mail address: lyn@novonordisk.com (L. Ynddal).

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mass spectrometer is a new approach and may prove to be useful in fast, simultaneous quantitation and identification of drug substances and metabolites.

Dextromethorphan (DMP) and its two main metabolites [dextrorphan (DP) and 3-methoxymorphinan (3-MM)] were selected as model compounds. Simultaneous determination of DMP and up to four of its metabolites using HPLC separation has previously been reported. A number of sample clean-up procedures such as liquid–liquid extraction [8,9], off-line solid-phase extraction [10] and on-line non-TFC column extraction [11] were used. As detection principles fluorescence were employed by Jones et al. [8], Bendriss et al. [10] and Härtter et al. [11], and tandem mass spectrometry (MS) was reported by Vengurlekar et al. [9]. Run times for the HPLC separations were reported to be between 11 and 30 min. Härtter et al. [11] reported an assay time of 15 min including an on-line clean-up step.

In the present paper a method for simultaneous quantitation of drug molecules combined with metabolite identification and semi-quantitative determination in rat plasma samples is described. The method employ on-line TFC and subsequent HPLC hyphenated to a fast scanning mass spectrometer. Total assay time including TFC clean-up, HPLC separation and MS detection are between 3.5 and 5 min. DMP is used as the model drug substance and DP and 3-MM are considered as unknowns. For proof of methodology real life rat plasma samples are assayed for dextromethorphan and subsequently searched for metabolites.

2. Experimental

2.1. Materials and reagents

Dextromethorphan was purchased from Sigma (St. Louis, MO, USA). Dextrorphan and 3-methoxymorphinan were purchased from Ultrafine Chemicals (Manchester, UK). Deionised water was obtained from a Milli-Q system (Millipore, Molsheim, France). HPLC-grade methanol was from Rathburn (Walkerburn, UK). Formic acid was analytical grade from Sigma–Aldrich (Steinheim, Germany). Micro titer 96 (MT96) well plates, (polypropylene; 0.3 ml) were obtained from Nunc (Roskilde, Denmark) and Sealing Tape Pads film for 96 well plates was from 3M (St. Paul, MN, USA). Cesium Iodide (CsI) was from Sigma and the synthetic peptide, ALILTLVS (Sex determining inhibitor) was from Bachem (Bubendorf, Switzerland).

2.2. Instrumentation

The TFC–LC–MS–MS system consisted of a CTC HTS PAL autosampler (Cohesive Technologies, Franklin, MA, USA) equipped with a 100-μl syringe, a Cohesive 2300 System equipped with two binary pumps and a valve interface module (VIM) controlled by the Aria OS software package (Cohesive Technologies). The system was configured as described by Herman [4], except that the loop on valve 1 was 100 μl. The mass spectrometer was an API QSTAR Pulsar i controlled by The Analyst QS software package (MDS Sciex, Concord, Canada). Metabolite information was extracted by the software package Metabolite ID (MDS Sciex). For sample preparation an Eppendorf centrifuge 5415C (Eppendorf, Hamburg, Germany) was used.

2.3. Solutions, calibration standards and quality controls

All stock solutions of analytes were made in methanol at 1.00 mg/ml (stored frozen, −20 °C). Working solutions were made fresh by dilution with methanol. Calibration standards and quality control (QC) samples were prepared in rat plasma. To 100 μl rat plasma was added 90 μl of water and 10 μl of working solution containing the analytes of interest. Calibration standards in the range 0.10–3000 ng/ml were prepared. QC samples were spiked to plasma concentrations of 1.00, 10.0 and 100 ng/ml. All concentrations are given in mass units as free base.

2.4. Sample preparation

Aliquots of 100 μl plasma samples were added to 100 μl 10% methanol in water and mixed. Calibrators, QC samples and plasma samples were centrifuged at 16 600 *g* for 15 min (Eppendorf centrifuge 5415C). The supernatant (ca. 170 μl) was transferred to MT 96 well plates and covered by Sealing Tape Pads prior to injection.

Table 1
Chromatographic conditions for the on-line Cyclone TFC clean-up and subsequent X-Terra MS C_8, 20×2.1 mm, 3.5 μm, separation applied prior to QSTAR data acquisition. Total run time 5.00 min

Load pump			Valves		Elution pump			Comment
Time (min)	Flow (ml/min)	% B	Valve position	Tee	Flow (ml/min)	Gradient	% B	
0.00	5.00	0	Load	Out	0.50	Step	0	Load sample and wash matrix to waste
0.50	0.15	0	Inject	In	0.85	Step	0	Transfer analytes and focus on HPLC column
2.50	2.00	95	Inject	Out	0.60	Step	10	Elution, fill loop, wash load system
2.67	2.00	95	Inject	Out	0.60	Ramp	85	Gradient elution, fill loop, wash load system
4.17	0.25	95	Load	In	0.25	Ramp	95	Valves cleaned
4.20	0.25	95	Inject	Out	0.25	Ramp	95	Valves cleaned
4.23	0.25	95	Load	In	0.25	Ramp	95	Valves cleaned
4.27	0.25	95	Inject	Out	0.25	Ramp	95	Valves cleaned
4.30	0.25	95	Load	In	0.25	Ramp	95	Valves cleaned
4.33	0.25	95	Inject	Out	0.25	Ramp	95	Valves cleaned
4.37	5.00	0	Load	Out	0.50	Step	0	Re-equilibrate system
5.00	5.00	0	Load	Out	0.50	Step	0	Re-equilibrate system

2.5. Chromatographic conditions

The TFC column was a Cyclone, 50 × 1 mm, 50 μm (Cohesive Technologies) and the HPLC columns were either a SpeedROD RP 18-e, 50 × 4.6 mm (Merck, Darmstadt, Germany) or an X-Terra MS C_8, 3.5 μm, 20 × 2.1 mm (Waters, Milford, MA, USA). Column temperature was ambient. The injection volume was 50 μl.

The mobile phases were: (A) methanol–0.05% formic acid (5:95, v/v) and (B) methanol–0.05% formic acid (95:5, v/v).

2.6. Assay procedure

The TFC and HPLC method consisted of five steps. Step 1: The sample was loaded onto the TFC column with an aqueous mobile phase (100% A). The elution pump delivered the same mobile phase composition. Step 2: The trapped analytes were transferred from the TFC column to the HPLC column and focused on top of the HPLC column. The transfer of analytes was done by back-flushing the TFC column with 100 μl 95% B stored in the loop of valve 1. The transfer eluent was diluted by an aqueous mobile phase (ratio 15:85) in a tee connection inside valve 2 and subsequent focused on the analytical column. Step 3: Gradient elution of analytes and re-fill of loop of valve 1. Step 4: Cleaning of valves and tubing. Step 5: System re-equilibration. The detailed assay procedure appears in Table 1.

2.7. Mass spectrometry

The mass spectrometer was equipped with a Turbo-Ionspray interface and operated in the positive mode (5000 V). Interface temperature was 350 °C and zero air was used as nebuliser and drying gas and was set at 3 and 6 bar, respectively. The time-of-flight (TOF) mass analyser was calibrated daily using CsI and ALILTLVS.

2.8. Rat plasma samples

Plasma samples were obtained from Sprague–Dawley rats dosed orally (gavage) with 2.27 mg DMP/kg body mass. EDTA blood samples were drawn by heart puncture at 15, 30, 60, 90, 120, 240 and 360 min post dose. Plasma was separated and stored frozen (<−20 °C) until analysis.

3. Results and discussion

3.1. TFC columns and conditions

TFC columns packed with C_8 or C_{18} bonded silica based or styrene–divinylbenzene copolymer columns

were tested for recovery and memory effects. For loading mobile phases of methanol mixed with water, 0.05% or 0.1% formic acid were evaluated. Methanol mixed with 0.05% formic acid gave the best signal intensity and chromatographic separation. Spiked plasma samples and solutions in 5% methanol was loaded under turbulent conditions with 100% A. Transfer was done with either 60% or 95% methanol mixed with water, 0.05% or 0.1% formic acid and with loop volumes of 50, 100 or 200 μl. Best recovery (ca. 60%) and no memory effect was obtained on the styrene–divinylbenzene column (Cyclone) using a loop volume of 100 μl on valve 1 and 95% B.

3.2. HPLC columns and conditions

For the development of fast TFC–HPLC screening assays the monolithic columns (e.g., SpeedROD RP 18-e) are first choice as they retain separation power at a very high flow-rate and with acceptable back pressure [12,13]. Using the Cohesive TFC system in dual column mode the use of a monolithic column as the analytical column allows high flow-rates in the transfer step compared to a column packed with 3.5 μm particles, thus reducing the transfer time and thereby total assay time. Transfer time using monolithic columns may be reduced to only 15–20 s compared to the transfer time for packed columns typically between 60 and 120 s. In the methods employed the transfer flow and time using the monolithic column was 1 ml/min and 26 s compared to the packed column at only 0.15 ml/min and 120 s. However, it should be noted that in this case the dimensions of the columns differ in favour of the large I.D. 4.6 mm of the monolithic column to the narrow bore I.D. of 2.1 mm of the packed column.

3.3. HPLC dimensions and sensitivity

Using the monolithic column fast transfer and subsequent focusing on the front end of the column was unproblematic using the conditions described. No bleeding through the transfer phase was observed and the fast gradient applied resulted in baseline separation of the metabolites, DP (t_R 1.8 min) and 3-MM (t_R 2.3 min). DMP and 3-MM co-elute with a peak separation in time of only 5 to 8 s. The high flow-rates applied (4 ml/min) resulted in a dilution of the analytes resulting in a limit of quantitation (LOQ) for DMP at 5 ng/ml. The post column split flow to the MS interphase was kept at 300 μl/min. The LOQ was unacceptable high as lower levels of parent drug and metabolites were expected in the rat plasma samples. In order to achieve better sensitivity an alternative column was employed.

The X-Terra MS C_8, 20×2.1 mm, 3.5 μm particles was found suitable for the assay. The separation pattern (Fig. 1) was similar to that obtained on the monolithic column. The column flow-rates were decreased to only 1 ml/min during the transfer phase and 0.6 ml/min during the elution phases of the assay (Table 1). To avoid break through of analytes on the X-Terra column the aqueous mobile phase used for the load and transfer phases was changed to 100% A. Compared to the monolithic system the runtime was increased by 90 s to a total runtime of 5.00 min on the packed column. However, the lower internal diameter of the column and the lower flow-rate applied resulted in an increase in both the concentration of the analytes in the eluent and in the fraction led to the mass spectrometer thus resulting in a lower limit of quantitation and identification. The limit of quantitation for DMP was decreased from 5 to 0.5 ng/ml plasma, equivalent to 12.5 pg DMP injected. The post column split allowed 300 μl/min to the MS interphase.

3.4. Mass spectrometer

For simultaneously acquisition of both quantitative and qualitative data an information dependent acquisition (IDA) method was employed. The survey scan

Table 2
Method parameters for an information dependent acquisition method used for simultaneous quantitative and qualitative data acquisition on a QSTAR Pulsar i mass spectrometer

Parameter	Setting
TOF ion range (*m*/*z*)	100–300
Product ion range (*m*/*z*)	100–300
For ion intensity above (cps)	10
Switch after (No. of spectra)	1
Exclude former target ions for (s)	0
Ion tolerance (μ)	50
Accumulation time (s)	0.8
Pulsar frequency (kHz)	9.986
Enhance all	Selected
Cycle time (s)	4.0004

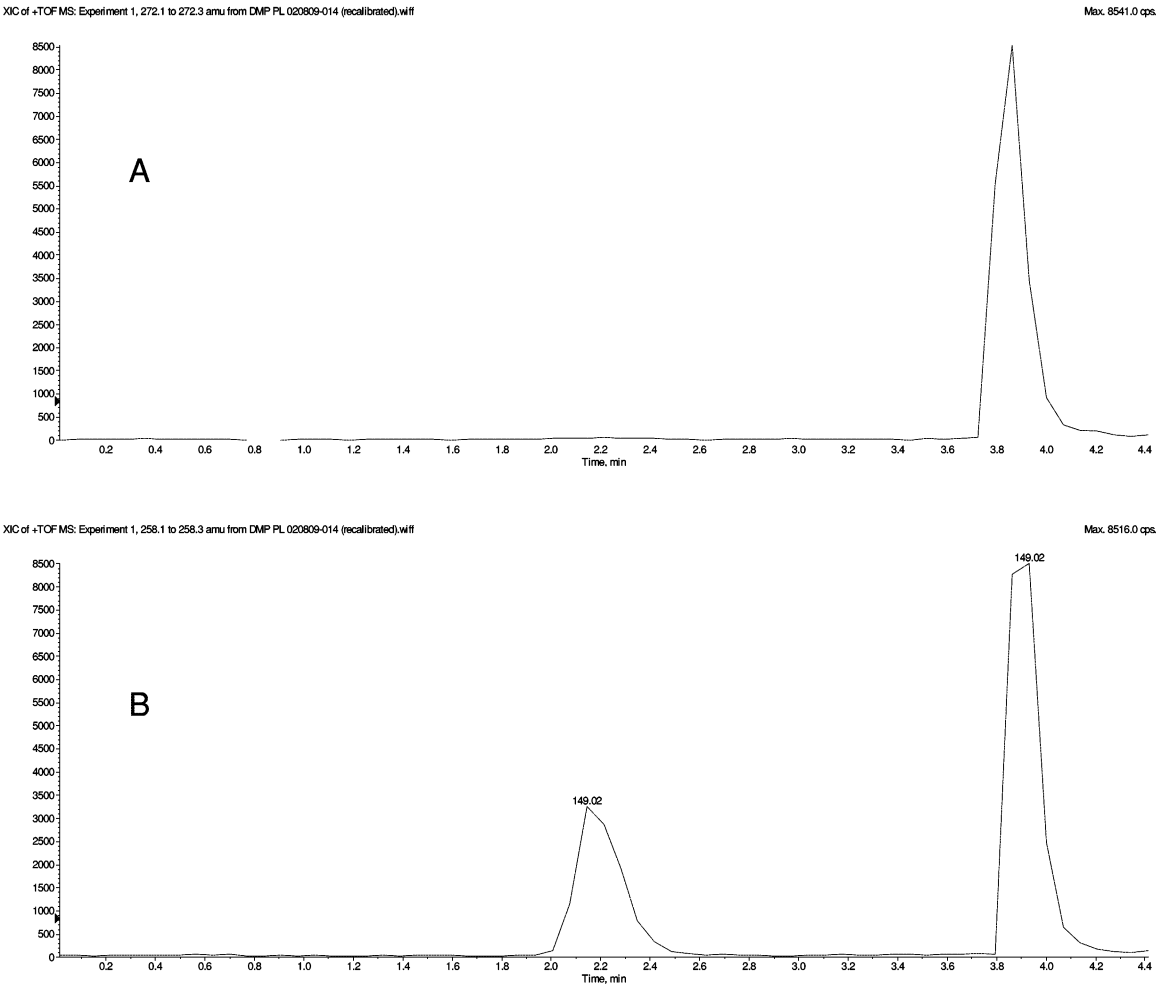

Fig. 1. TOF-MS extracted ion chromatograms of (A) dextromethorphan (Rt. 3.90) and (B) dextrorphan (Rt. 2.15) and 3-methoxymorphinan (Rt. 3.95). Spiked rat plasma, all analytes 75 ng/ml. Chromatogram extracted after on-line Cyclone™ TFC clean-up and separation on an X-Terra™ MS C8 (2.1×20 mm) particle based HPLC column. Data acquired through an Information Dependant Acquisition method applied on a QSTAR™ mass spectrometer.

was TOF-MS and the dependent scans were four product ion scans. The IDA method description appears in Table 2.

The duty cycle of the IDA method was 4 s. Using the monolithic column with high flow-rates the peak width was about 10–15 s at base line allowing only 3–4 scans to be done across the peaks. The quality of the data acquisition on these narrow peaks would be poor due to the sparse number of data points. The lower flow applied on the packed column method resulted in peak width at base line at 25–30 s allowing 6–8 scans across the peaks (Fig. 1). Six to eight data points per peak will result in adequate quality for qualitative data acquisition whereas quantitative data quality would be less acceptable as 10–15 data points is considered to be the minimum. The duty cycle could be shortened by reducing the number of product ion scans. The IDA method consisted of five steps each having an accumulation time of 0.8 s, so eliminating one or two product ion scans would result in duty cycles of 3.2 and 2.4 s, respectively. The disadvantage of this is that co-eluting unknown interferences could pass the threshold for

the dependent scan and thus compete for the analytes of interest. The reduced duty cycle is presently under investigation. It is beneficial to enter known interferences into the exclusion list and known or guessed metabolites into the inclusion list of the IDA experiment for improvement of the data acquisition.

3.5. Metabolite identification

The data were analysed by the Metabolite ID software package. A predefined list of target transformations for the most common phase I metabolites was selected and the data files were analysed for the appearance of peaks having the related mass changes. In the present investigation only peaks having extracted ion chromatogram signal intensities above 1000 counts per second (cps) had product ion scans. Below 1000 cps the product ion scans were empty. DMP and 3-MM had a characteristic product ion of *m*/*z* 171 whereas DP had a product ion of *m*/*z* 157 indicating the loss of a methyl group in the fragment compared to both DMP and 3-MM. For DMP and 3-MM the lower limits for product ion scans were 10 ng/ml, equivalent to 250 pg injected. For DP the lower limit for product ion scans was 50 ng/ml, equivalent to 1.25 ng injected. For samples with analytes below these concentrations the metabolite identification relied only on accurate mass measurements.

3.6. Accurate mass measurements

The TOF mass analyser of the mass spectrometer is capable of delivering mass measurements with accuracy down to 5 ppm (parts-per-million) or better [14] thus allowing elemental composition of the detected species. Such accurate mass measurements require typically 10–20 scans for adequate ion statistics and using multiple channel averaging. In the present expe-

Table 3
Mass errors on acquired and re-calibrated data from plasma calibrator 75 ng/ml, QC sample 10 ng/ml and rat plasma sample 15 min post dose. Samples analysed by on-line Cyclone TFC sample clean-up and subsequent X-Terra MS C_8, 20 × 2.1 mm, 3.5 μm, separation applied prior to QSTAR Pulsar i data acquisition

Parameter	Dextromethorphan	Dextrorphan	3-Methoxymorphinan
Plasma calibrator 75 ng/ml			
Theoretical mass (H^+)	272.2008	258.1852	258.1852
Acquired mass (H^+)	272.2070	258.1905	258.1900
mu error	6.1088	5.2589	4.7589
ppm error	22.4420	20.3685	18.4320
Re-calibrated mass (H^+)	272.2008	258.1846	258.1843
Re-calibrated mu error	−0.0911	−0.6410	−0.9410
Re-calibrated ppm error	−0.3347	−0.4828	−3.6447
QC sample 10 ng/ml			
Theoretical mass (H^+)	272.2008	258.1852	258.1852
Acquired mass (H^+)	272.2023	258.1885	258.1884
mu error	1.4088	3.2589	3.1589
ppm error	5.1758	12.6224	12.2351
Re-calibrated mass (H^+)	272.2008	258.1860	258.1870
Re-calibrated mu error	−0.0911	0.7589	1.7589
Re-calibrated ppm error	−0.3347	2.9396	6.8127
Rat plasma sample 15 min post dose 2.27 mg/kg orally			
Theoretical mass (H^+)	272.2008	258.1852	258.1852
Acquired mass (H^+)	272.2012	258.1894	N/A
mu error	0.1088	4.1589	N/A
ppm error	0.4000	16.1081	N/A
Re-calibrated mass (H^+)	272.2005	258.1903	N/A
Re-calibrated mu error	−0.3911	5.0589	N/A
Re-calibrated ppm error	−1.4368	19.5939	N/A
Estimated plasma concentration (ng/ml)	6.2	6.6	N/A

riment the acquisition was done during an HPLC run and the number of spectra was set to one resulting in mass errors from 0.4 and up to 22 ppm. DMP was used as reference compound for internal mass re-calibration leading to a decrease in mass error to between −0.09 and 6.8 ppm. However, for very low signal intensities no improvement in mass errors was obtained.

However, the acquired accurate masses of the protonated ions of DMP, DP and 3-MM were used for estimating the elemental composition. Knowing the formula of the drug dosed (DMP) suggested compounds with elemental compositions not possible were easily de-selected thus leaving only the de-methylated metabolites. If product ion scans had been available the metabolite fragment pattern could have been used for both improved identification and suggestion of site of metabolism. Mass errors on analytes appear in Table 3.

3.7. Assay performance

The calibration curve was constructed by quadratic regression of area versus concentration (weighted $1/x$),

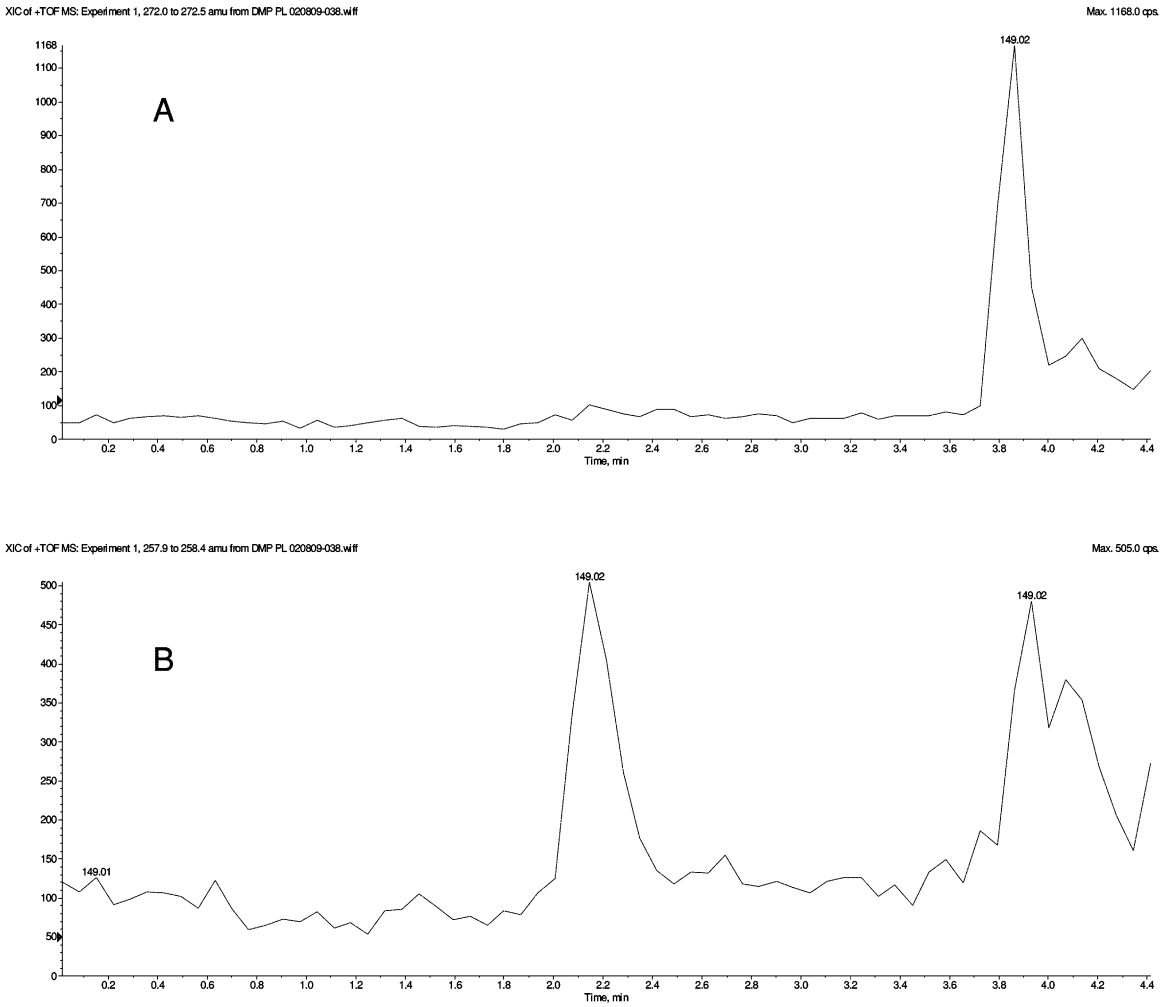

Fig. 2. TOF-MS extracted ion chromatograms of (A) dextromethorphan (Rt. 3.86) and (B) dextrorphan (Rt. 2.14) and traces of 3-methoxymorphinan (Rt. 3.93). Real life rat plasma obtained 15 minutes post dose after per orally dosing of dextromethorphan (2.27 mg/kg). Chromatogram extracted after on-line Cyclone™ TFC clean-up and separation on an X-Terra™ MS C8 (2.1×20 mm) particle based HPLC column. Data acquired through an Information Dependant Acquisition method applied on a QSTAR™ mass spectrometer.

Table 4
Concentrations and accuracies of dextromethorphan calibration standards and QC samples for the on-line Cyclone TFC clean-up and subsequent X-Terra MS C_8, 20×2.1 mm, 3.5 μm, separation applied prior to QSTAR data acquisition

Nominal concentration (ng/ml)	Calibration standards		QC sample No. 1		QC sample No. 2	
	Concentration (ng/ml)	Accuracy (%)	Concentration (ng/ml)	Accuracy (%)	Concentration (ng/ml)	Accuracy (%)
0.50	0.498	99.6				
1.00	1.03	103	1.09	109	1.03	103
5.00	5.10	102				
10.0	9.37	93.7	8.26	82.6	9.11	91.1
25.0	25.4	102				
50.0	50.6	101				
75.0	74.4	99.3				
100	99.9	99.9	97.1	97.1	91.3	91.3

$y = 3.86x^2 + 1.4 \cdot 10^3 x + 1.29 \cdot 10^3$. The dynamic range of DMP was estimated to 0.5–100 ng/ml and coefficient of determination was better than 0.99. The co-assayed QC samples showed accuracies within 80–120%. Results and accuracies of calibration standards and QC samples appear in Table 4.

3.8. Assay of in vivo rat plasma samples

EDTA rat plasma samples were assayed and DMP concentrations estimated. DMP was quantitated in all samples from 15 to 360 min. The data files were analysed by Metabolite ID for phase I metabolites. DP was identified and quantitated in all samples, whereas 3-MM was only observed in few samples but signal intensities were below the LOQ. A rat plasma sample chromatogram is shown in Fig. 2. The obtained concentration–time profiles of DMP and DP are shown in Fig. 3, one data point represents one animal. Due to the low concentrations and thereby the low signal intensities product ion scans were not acquired for DMP and DP. Accurate mass measurement was used for calculation of possible elemental composition and thus the compound identification.

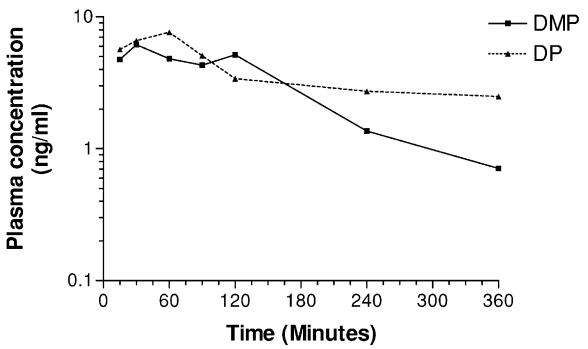

Fig. 3. Concentration-time plots of dextromethorphan (DMP) and dextrophan (DP) in rat plasma. Sprague Dawley rats were dosed per orally (gavage) with dextromethorphan. The dose was 2.27 mg/kg body weight. Plasma samples were collected at predefined time points from 15 to 360 minutes post dose.

4. Conclusion

A rapid and sensitive TFC–HPLC–MS method for the quantitative determination of dextromethorphan and the simultaneous identification of the *O*- and *N*-demethyl metabolites of dextrormethorphan in rat plasma was developed. Total run time including on-line TFC sample clean up and HPLC separation was 5.00 min. The lower LOQ and identification was 0.5 ng/ml corresponding to 12.5 pg injected. For the first time a fully automated assay including sample clean-up using TFC–HPLC–TOF-MS has been demonstrated. The technique is used for simultaneous quantitative and qualitative determination of parent drug and metabolites in real life plasma samples. This methodology may be applied early in the drug discovery screening phase providing in vivo ADME information during lead optimisation.

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