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Liquid chromatography–tandem mass spectrometry determination of loperamide and its main metabolite desmethylloperamide in biological specimens and application to forensic cases

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Abstract

A liquid chromatographic mass spectrometric (LC/MS/MS) method has been developed for the determination of loperamide in whole blood and other biological specimens. The procedure involves liquid–liquid extraction of loperamide, desmethylloperamide and methadone-D_3 (internal standard) with butyl acetate. Confirmation and quantification was done by positive electrospray ionisation with a triple quadrupole mass spectrometer operating in multiple reaction-monitoring (MRM) mode. Two MRM transitions of each compound were established and identification criteria were set up based on the ratio of the responses between the two MRM transitions of each compound. The standard curves were linear over a working range of 0.1–500 μg/kg for all transitions. The limit of quantification was 0.1 μg/kg in whole blood. The repeatability and reproducibility within the laboratory expressed by relative standard deviation were less than 5 and 11%, respectively, and the accuracy was better than 9%. The method was developed to examine a feces sample from a child whose mother was suspected of Münchausen syndrome by proxy and it proved to be suitable for forensic cases being simple, selective and reproducible. The method was also applied for a case investigation involving a overdose of loperamide.

Keywords: Loperamide; Desmethylloperamide

1. Introduction

Loperamide (Fig. 1) is a widely used antidiarrhoeic opioid without a central effect that is available without prescription. Initial recommended dosage is 4 mg and the daily dosage in adults range from 4 to 16 mg. The plasma concentrations of loperamide in humans are very low making the quantitative determination in body fluids difficult. Peak plasma level of loperamide is reported at 2 μg/l [1], and investigations with single oral bolus of 16 mg loperamide showed peak plasma concentrations about 8 μg/l [2,3].

Only two papers have described analytical methods sensitive enough for determination of loperamide and metabolite(s) (desmethyl- and didesmethyl-loperamide) in plasma [2,3]. After oral administration of 16 mg loperamid the maximum desmethylloperamide plasma concentrations were the same magnitude as observed for loperamide about 5 μg/l. The half-life of desmethylloperamide was longer than that of the parent compound, while the concentration of didesmethylloperamide was the lowest of all [3]. Both methods analysed organic extracts of alkaline plasma by high-performance liquid chromatography-atmospheric-pressure ionisation mass spectrometry [2,3]. The Department of Forensic Chemistry at the Institute of Forensic Medicine performs the toxicological analysis on the driving and criminal cases (offences) in Denmark and the post-mortem cases of East Denmark as requested by the police. These analyses involve complex specimens, such as whole blood, stomach contents, liver/muscle tissue and feces.

This paper concerns the development of a LC/MS/MS method, using positive ESI with a triple quadrupole mass spectrometer for the determination of loperamide and desmethylloperamide in whole blood and other complex

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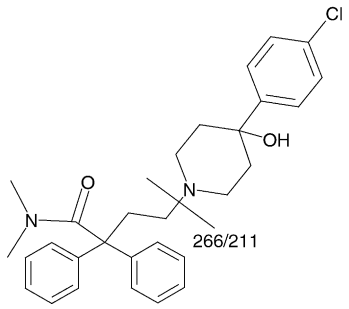

Fig. 1. Chemical structure of loperamide with main fragmentation point.

biological specimens in connection to forensic cases. Two cases are studied; a case of suspected Münchausen syndrome by proxy that required a highly sensitive and selective determination in a feces sample and a case involving a fatal overdose of loperamide.

2. Experimental

2.1. Chemicals and materials

Loperamide and desmethylloperamide was acquired from Jansson-Cilag A/S. Solvents of chromatographic grade were used for extraction and analysis. As internal standard (IS) DL-methadone-D_3 (312.4 g/mol) with a purity 99% from Cerilliant, USA, was used. All other reference substances were of high purity. Sodium hydroxide was of analytical grade from Merck. Control whole blood was obtained from horses.

2.2. Stock solutions and standards

Standards from 0.1 to 500 μg/l with 50 μg/l IS were prepared in mobile phase, while recovery standards were made from 0.1 to 500 μg/kg whole blood as described below. Biological samples were weighed prior to analysis.

2.3. Sample preparation

0.50 g whole blood or 0.5 g matrix homogenate was mixed with 20 μl 0.5 mg/l aqueous D_3-methadone. Other solid matrices, such as muscle and liver tissue were homogenized after dilution (1 + 2, w/v) with water (Stomacher 80, Lab-blender). The sample was extracted with 250 μl butyl acetate after adjusting pH with 50 μl 2 M NaOH. After centrifugation for 10 min the organic fraction was removed and evaporated to dryness at 40 °C and reconstituted in 100 μl mobile phase. Occasionally anhydrous sodium sulphate was added to improve separation of the phases, or samples were placed in a freezer for 10 min prior to centrifugation. Finally, the solution was transferred to an autosampler vial and 10 μl was injected into the chromatographic system.

2.4. Chromatography and MS conditions

For separation an Agilent 1100 series HPLC system consisting of binary pump, autosampler, and thermostatted column compartment was used with the following conditions: Zorbax SB-C18 column (2.1 mm × 30 mm, 3.5 μm). The mobile phase consisted of 30% aqueous acetonitrile, containing 10 mM formic acid, using a flow rate of 0.2 ml/min at 30 °C.

A Quattro LC and later a Quattro micro, tandem quadrupole mass spectrometer (Micromass) was coupled to the HPLC system. Data was acquired in the positive ion mode with an electrospray (ESI) source, using MassLynx software 3.5 and 4.0, and calculations used extracted ion chromatograms with QuanLynx. Mass spectrometer conditions (cone, lens voltage, collision energy, etc.) were optimised by direct infusion of the standard into the Z-spray ion source by a Harvard syringe pump (Table 1). Multiple-reaction monitoring (MRM) analysis was used for data collection (Table 1). The source and desolvation temperatures were 120 °C and 350 °C, respectively. Ion suppression experiment was performed by injecting Lop and De-lop through the Harvard pump into a T-piece with mobile phase. Suppression was examined in extracts of whole blood within a 100-min run.

3. Results and discussion

3.1. Chromatography and MS conditions

Methadone-D_3 was chosen as the internal standard due to its similar chemical structure and its well-described behaviour in the system including high recovery [5]. The absolute recovery was 60% for methadone in whole blood, 75% for loperamide (Lop) and 80% for desmethylloperamide (De-Lop) within the whole concentration range. The internal standard eluted at a retention time of 3.4 min, De-Lop at 4.5 min and Lop at 8.75 min. Screening of drug-free whole blood showed no endogenous interference at the retention times of the compounds and no ion suppression was observed on Lop or De-lop during a 100-min run after injection of whole blood extracts.

By ESI single-protonated molecular ions for Lop *m/z* 477 and de-Lop *m/z* 463 were produced, and their isotopes from

Table 1
MS/MS conditions for the Quattro LC (Quattro micro)

	Cone Energy (V)	Collision Energy 1 (V)	Collision Energy 2 (V)	Transition 1 MRM1	Transition2 MRM2	Ion ratio MRM2/MRM1
Loperamide	38 (37)	25 (15)	50 (38)	477→266	477→210	0.74
Desmethylloperamide	38 (37)	28 (25)	38 (47)	463→252	463→196	0.46
IS (D_3-Methadone)	30 (25)	15 (15)		313→286		

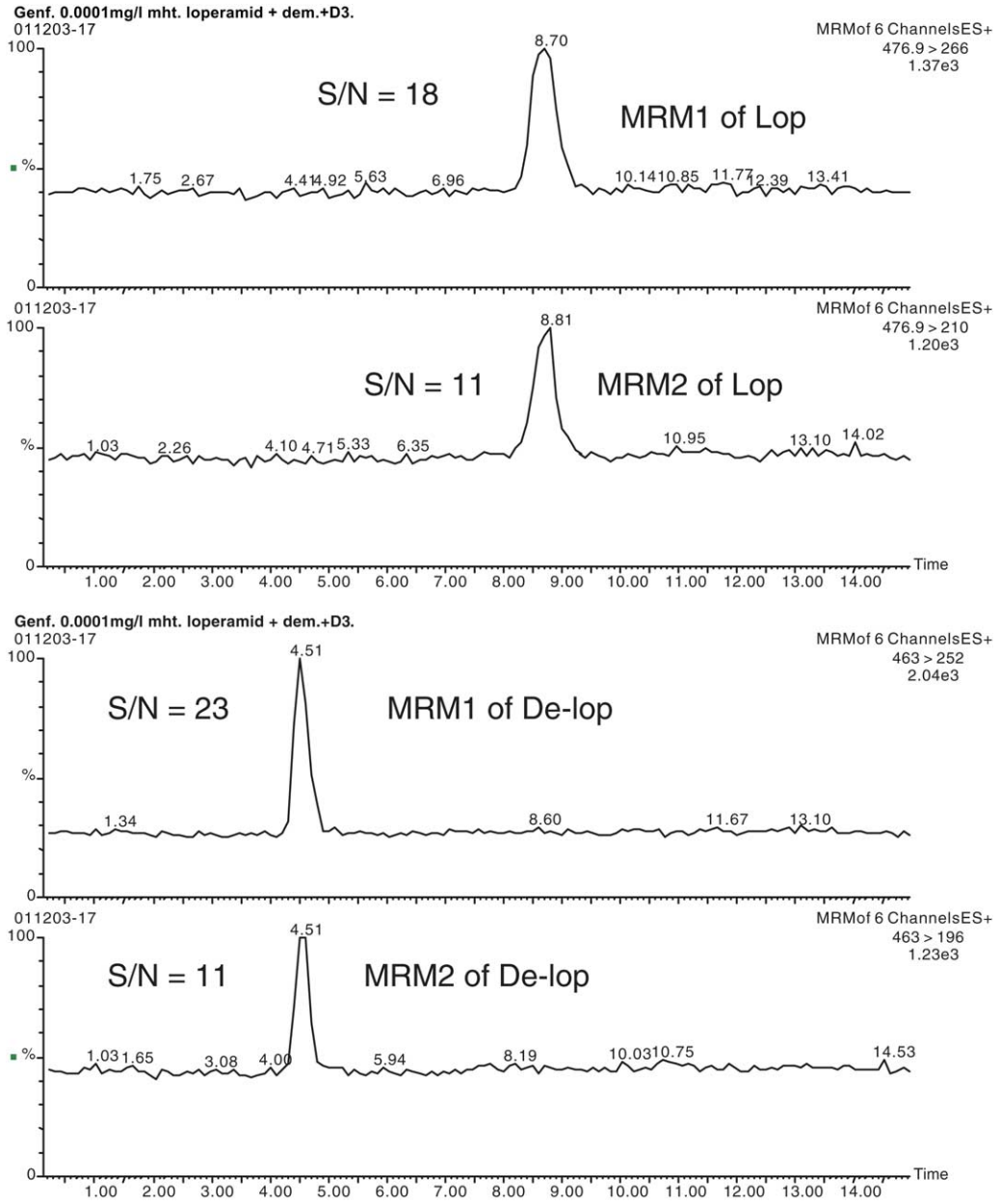

Fig. 2. LC/MS/MS chromatograms of spiked whole blood at 0.1 μg/kg of loperamide and desmethylloperamide, respectively. The absolute recovery of the internal standard was 60%. For each compound their two MRM transitions are shown with the signal-to-noise level (*S*/*N*).

Cl^{37} were also detected with a ratio of 1:3 as expected from theory. The main fragments in MS/MS mode were obtained by cleavage of the piperidyl bond and fragmentation of the pyrrolidine ring (Fig. 1). Two intense ion-pair transitions were chosen for identification and quantification of Lop and its main metabolite, De-lop (Table 1). As identification criteria both relative and absolute retention times can be used. The absolute retention time may have a maximum deviation of ±0.1 min or the relative time when using the internal standard may have a maximum deviation of ±2%. Furthermore, the ratio of the responses for MRM2 against MRM1 may have a maximum deviation of 5%. In other papers this deviation has been set to 20% [6], which is too wide for these substances. The ion ratio was stable in the instrument for over 2 years, the relative standard deviation of the ratio for loperamide was determined to be 3% and for De-Lop 5% (N = 6). Finally, concerning the identification criteria, detection of the metabolite, desmethylloperamide confirmed the intake of loperamide.

3.2. Linearity and limit of quantification

The standard curves of pure standards made up in mobile phase (N = 12) were linear over a working range of 0.1–500 μg/kg for all four transitions (Table 1). The calibration graphs were derived by plotting the peak area ratio of

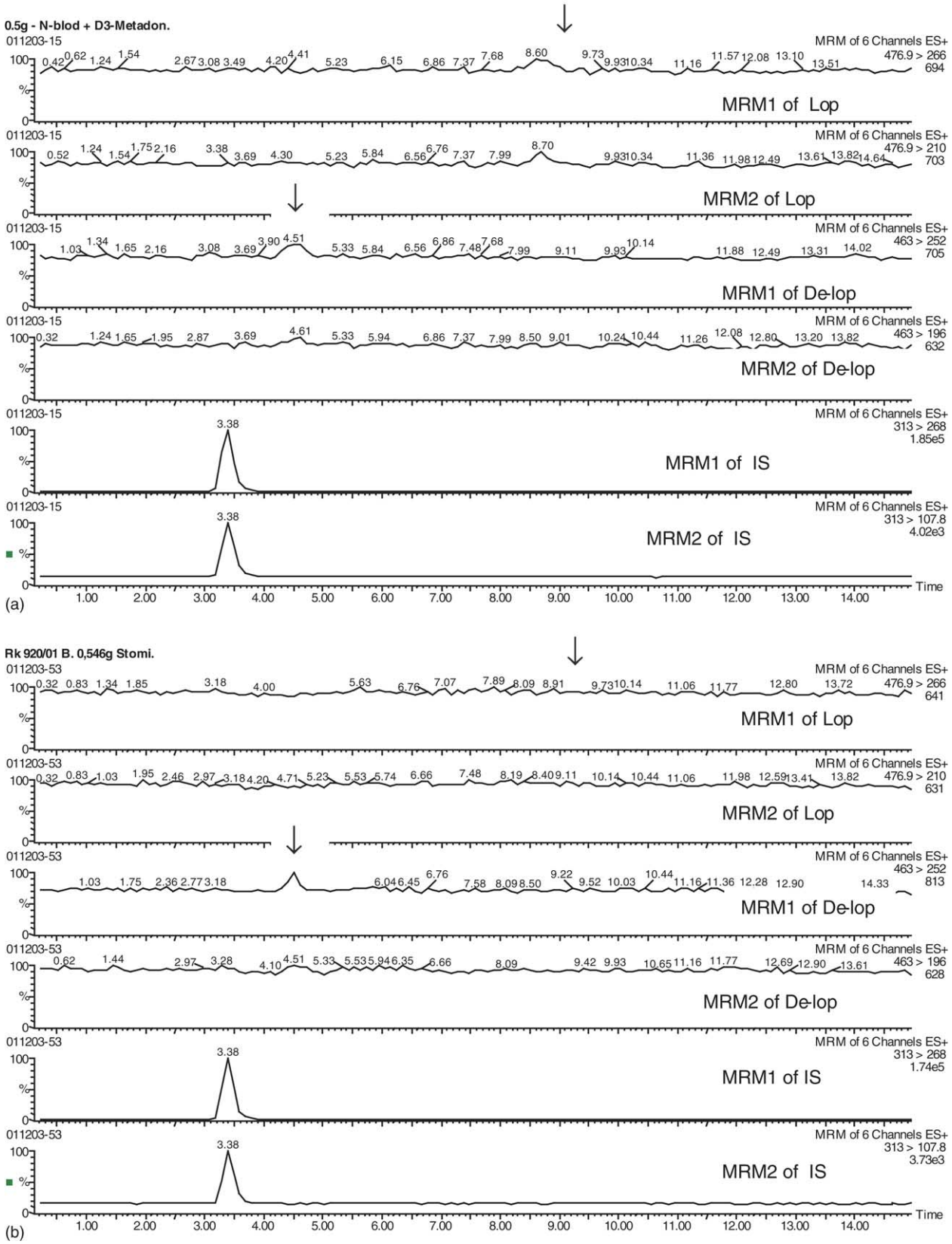

Fig. 3. (a) A typical LC/MS/MS chromatogram of blank whole blood with internal standard (recovery: 100% relative). *S/N* < LOD. (b) Chromatogram of an extracted feces sample from a child assumed to be poisoned with loperamide. The relative recovery of internal standard was 80% in the feces sample. The sample was found negative for loperamide and its main metabolite desmethylloperamide. The arrows indicate the retention time of De-Lop and Lop.

Table 2
Intra- and inter-assay precision and accuracy

	Concentration added (μg/kg)	Repeatability				Intra-day repeatability			
		Mean (μg/kg)	BIAS (%)	R.S.D. (%)	*N*	Mean (μg/kg)	BIAS (%)	R.S.D. (%)	*N*
Lop	0.500	0.472	−5.6	4.3	6	0.492	−9.0	11	5
	50.0	52.1	4.2	3.6	6	50.1	0.2	9.4	5
De-Lop	0.500	0.510	2.0	3.3	6	0.525	5.0	11	5
	50.0	52.1	4.2	3.0	6	50.1	0.2	6.4	5

N: number of samples.

Lop or De-Lop to the IS versus the whole blood concentration of Lop or De-Lop: linear regression, with 1/*x* weighting were used. The linear function of Lop was $52.1x - 0.011$ with a correlation coefficient (r^2) of 0.9982 (MRM1) and $19.3x - 0.004$ with $r^2 = 0.9983$ (MRM2), while for De-Lop it was $47.2x - 0.007$ with $r^2 = 0.9998$ (MRM1) and $16.1x - 0.002$ with $r^2 = 0.9998$ (MRM2).

The limit of quantification was 0.1 μg/kg for Lop and De-lop in whole blood as illustrated in Fig. 3. The limit of quantification was defined as the lowest concentration at which the signal-to-noise level (*S*/*N*) of the extracted sample was higher than 10. All four transitions fulfilled this *S*/*N* level. The sensitivity of transition 1 was two times higher than transition 2 of each compound as shown in Fig. 2. The limit of detection was about 0.02 μg/kg for each transition when defined as *S*/*N* > 3.

3.3. Precision and accuracy

The day-to-day variability and intra-day repeatability expressed by relative standard deviation were less than 5 and 11%, respectively, and the inaccuracy did not exceed 9% of both compounds as illustrated in Table 2.

The previously published LC/MS/MS methods were only developed for urine and plasma [2,3], while this method was applied to several other biological specimens, such as whole blood and tissues. However, the sample preparation principle and MS/MS conditions were similar.

3.4. Case investigations

The method was developed to examine a feces sample from a child whose mother was suspected of Münchausen

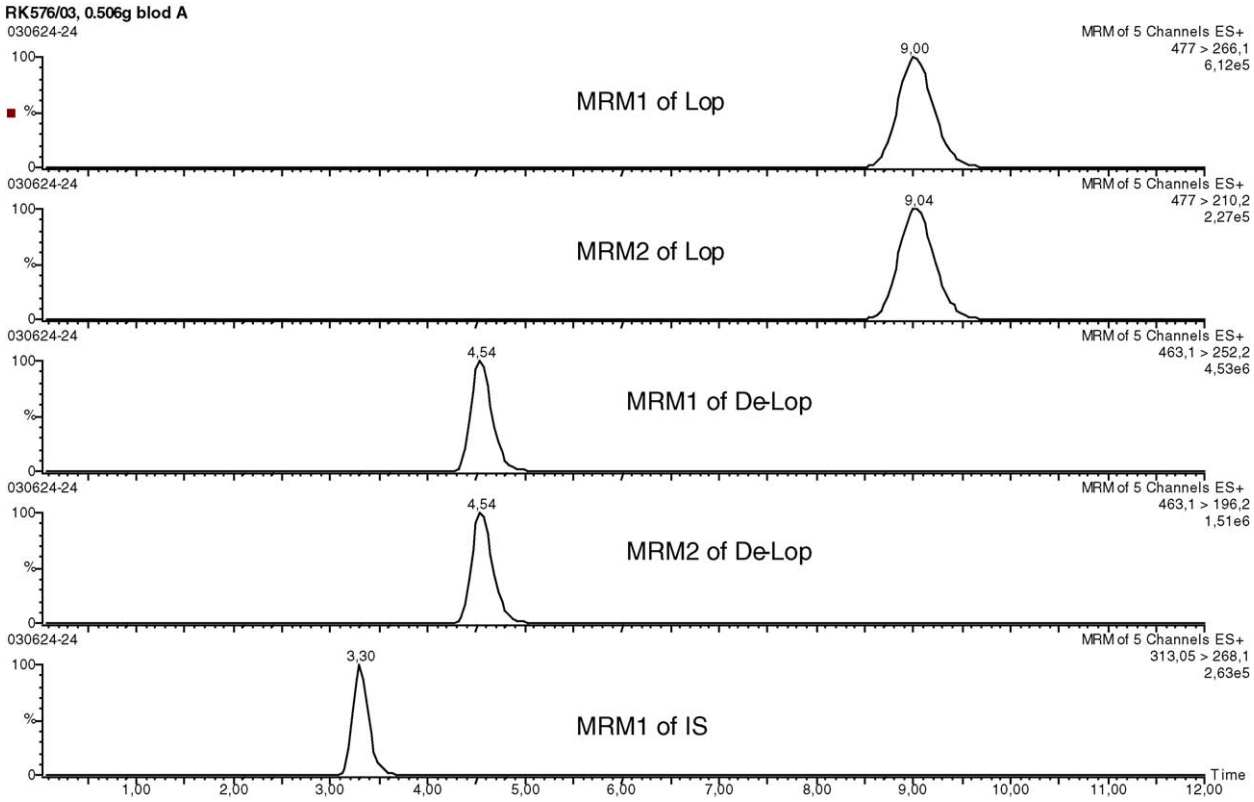

Fig. 4. LC/MS/MS chromatograms of extracted post-mortem blood from a 34-year-old man. The recovery of internal standard was 100% in the sample. The positive sample contained 0.084 mg/kg loperamide and 0.38 mg/kg desmethylloperamide. The identification criteria were fulfilled. This analysis was performed on the Quattro micro mass spectrometer. Loperamide was also detected in the liver tissue.

syndrome by proxy (MSBP). MSBP is defined as the intentional production or feigning of physical or psychological signs or symptoms in another person who was under the individual's care for the purpose of indirectly assuming the sick role [7]. The involved child had bowel stomia and a feces sample was received from her for the investigation. The police had confiscated empty packs of Dulcolax® that contains bisacodyl, and Imodium® that contains loperamide. The screening methods detect bisacodyl, but not Lop at therapeutic levels. Approximately 50% Lop is hepatically metabolised to inactive glucuronides and 40% is excreted unchanged in the feces and 1% in urine [1,8] indicating that the feces sample was an excellent matrix for this investigation. The chromatogram of the extracted feces is shown in Fig. 3 together with the chromatogram of an extracted blank whole blood sample. The recovery of the internal standard was 80% in the feces relative to whole blood, which is acceptable for this matrix. Based on the developed method, the laboratory concluded that Lop and De-lop were not detected in the feces sample because none of the substances were detected (see arrows in ion traces in Fig. 3).

Another case involving a 34-year-old man, who died alone at home, was studied, where the routine analysis had detected Lop in the liver tissue by basic screening, using GC/MS. The described LC/MS/MS method was therefore applied to the post-mortem blood of the 34-year-old man and an extract is shown in Fig. 4. The relative recovery of the IS in the whole blood sample was 100% and the identification criteria were fulfilled. The post-mortem blood sample contained 0.084 mg/kg Lop and 0.38 mg/kg De-lop, while the liver tissue contained 0.87 mg/kg Lop and 3.8 mg/kg De-lop, respectively. The relative recovery of the IS in the liver sample was 70%. It is concluded that the case involved a fatal overdose of loperamide.

Loperamide poisoning has been described in 19 infants with severe abdominal distension and paralytic ileus as a result of being given Imodium drops in Pakistan [4], however, the concentration of Lop was not determined. Lop does not exhibit the typical morphine-like effects of its structural analogue, diphenoxylate, except in very high doses. Adverse effects associated with therapeutic doses include dizziness, drowsiness and dry mouth [1]. A number of children under 4 years of age who were believed having been given therapeutic doses of the drug have developed symptoms that included irritability, delerium, respiratory depression, sedation, hypotonia and obtundedness, but all recovered [1]. However, in the case reported here no indication of his symptoms was given before death, because he died at home alone. According to the medical file the man suffered from AIDS; whether this had any influence on the outcome is uncertain.

This analysis was performed by a Quattro micro mass spectrometer, because the laboratory had exchanged the Quattro LC mass spectrometer. In this exchange it was observed that the ion ratio of Lop and De-lop varied with instrument type due to change in collision chamber between the instruments. However, comparable sensitivity between instruments was obtained. In the future, a deuterated loperamide as internal standard is recommended, however, it is not yet available on the market. Commercially, the two methods are applied to forensic cases in the laboratory, and the principles are now being used with other potent drugs, such as fentanyl and LSD.

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