Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 795 (2003) 41–53
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous screening for 238 drugs in blood by liquid chromatography–ionspray tandem mass spectrometry with multiple-reaction monitoring

M. Gergov*, I. Ojanperä, E. Vuori

Department of Forensic Medicine, University of Helsinki, P.O. Box, 40, FIN-00014 Helsinki, Finland

Received 30 January 2003; received in revised form 1 May 2003; accepted 20 June 2003

Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method is presented for the qualitative screening for 238 drugs in blood samples, which is considerably more than in previous methods. After a two-step liquid–liquid extraction and C_{18} chromatography, the compounds were introduced into a triple quadrupole mass spectrometer equipped with a turbo ion spray ion source operating in the positive ionization mode. Identification was based on the compound's absolute retention time, protonated molecular ion, and one representative fragment ion obtained by multiple reaction monitoring (MRM) at an individually selected collision energy of 20, 35, or 50 eV. The limit of detection (LOD) for the majority of the compounds (80%) was ≤0.05 mg/l, ranging from 0.002 mg/l (e.g., antihistamines) to 5 mg/l (acidic compounds), and for malathion it was 10 mg/l. The LOD values were sufficiently low to allow the majority of compounds to be detected at therapeutic concentrations in the blood.

Keywords: Screening; Multiple-reaction monitoring; Drug screening

1. Introduction

Screening for a wide range of toxicologically relevant compounds in biological samples continues to be a challenge for forensic and clinical toxicology laboratories. None of the existing techniques, thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), liquid chromatography (LC), or gas chromatography–mass spectrometry (GC–MS), shows superior performance in this area. Liquid chromatography–mass spectrometry (LC–MS) is current and very promising tool for comprehensive screening analysis. Marquet [1] has recently evaluated the suitability of LC–MS for toxicological screening and concluded that it is probably as efficient as GC–MS or LC with diode array detection and complementary to those techniques. LC–MS provides three alternatives: (1) with a single-stage mass spectrometer (LC–MS) in the scan mode and applying in-source collision-induced dissociation (in-source CID), the sample is screened at variable orifice voltages, and all peaks exceeding the preset criteria for intensity are identified by comparison of their spectra with in-source CID spectrum libraries; (2) with a tandem mass spectrometer (LC–MS–MS) and data-dependent experi-

*Corresponding author. Tel.: +358-9-1912-7485; fax: +358-9-1912-7518.
E-mail address: merja.gergov@helsinki.fi (M. Gergov).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00498-7

ment (DDE), the sample is first screened in the scan mode, then in an automatically started second run, the MS–MS spectrum is acquired from all ions exceeding the preset area threshold limit; (3) with LC–MS–MS instrument and multiple reaction monitoring (MRM), samples are monitored for a large number of previously selected compounds.

Three recent studies describe screening methods using LC–MS with in-source CID. In the papers by Marquet et al. [2] and Saint-Marcoux et al. [3], four separate spectra were generated at the same time by changing the ionization polarity and orifice voltage during the run. The positive spectra obtained (at +20 and +80 V) were summed, as well as the negative spectra (−20 and −80 V), and both sum spectra were compared to the spectrum libraries. In another application, by Weinmann et al. [4], the orifice voltage was varied between three voltages in the positive mode and the three spectra simultaneously obtained were then separately searched against the spectrum library. In the third application, Rittner et al. [5] used two different voltages, and searched both spectra separately. A limitation of these in-source CID approaches is that the origin of the spectra is unknown, because in complex biological extracts, compounds may co-elute, causing summed spectra that are difficult to resolve.

LC–MS–MS with DDE is an effective tool for simultaneous screening and identification. This technique has been applied by Fitzgerald et al. [6] with a quadrupole ion trap instrument and by Decaestecker et al. [7] with a quadrupole time-of flight instrument. These instruments allow screening and identification during one chromatographic run; however, with a triple quadrupole instrument, the identification step requires a second run. In a different type of approach, the present authors used a single time-of-flight mass spectrometer (LC–MS-TOF) [8] to screen 433 compounds by their accurate mass.

A benefit of tandem over single MS is better specificity, because the daughter ions originate only from the selected parent ion. Moreover, in all applications based on screening in the scan mode, the sensitivity may be too low to detect small peaks on the total ion chromatogram, and those peaks will therefore already be missed in the screening step. Because LC–MS–MS with MRM offers better sensitivity and selectivity than does the scan mode, this approach has been used for the screening and quantitation of various drug groups which comprise a limited number of compounds [9,10]. In an earlier application at our laboratory [11], an additional confirmation step added to the procedure used DDE and library search of the product ion spectra.

Our object was to show that by using LC–MS–MS with MRM, it is technically possible simultaneously to screen for a significantly higher number of compounds than has been reported earlier, and in a very simple, rugged, and sensitive manner.

2. Experimental

2.1. Materials

All standard compounds came from various pharmaceutical companies and all were of pharmaceutical purity. Acetonitrile and methanol (HPLC grade) were purchased from Rathburn (Walkerburn, UK), ammonium acetate (p.a.), formic acid (p.a.), dichloromethane, isopropanol, and tris(hydroxymethyl)-aminomethane (Tris, p.a.) from Merck (Darmstadt, Germany). Purified water was generated with an Alpha-Q water purification system from Millipore (Bedford, MA, USA).

2.2. Instrumentation

HPLC separations were carried out with PE Series 200 LC–MS Pumps and Autosampler using a vacuum degasser. A Genesis C_{18} column (100 mm×2.1 mm, particle size 4 μm, Jones Chromatography, Hengoed, UK) was used for separation and a Purospher RP-18 LiChro Cart 4-4 (40 mm, particle size 4 μm, Merck, Darmstadt, Germany) as a guard column. The mass spectrometric analysis was performed in positive ion mode using a PE Sciex API 365 triple stage quadrupole LC–MS–MS (Concord, ON, Canada) instrument equipped with a PE Sciex Turbo Ion Spray interface.

2.3. Methods

2.3.1. Standard solutions

Separate stock solutions were prepared at a concentration of 1.0 mg/ml of free compound or salt in

methanol–water (1:1): 32 standard mixtures were made from the stock solutions, each containing four to nine compounds at a concentration of 10 μg/ml (basic compounds) or 100 μg/ml (acidic compounds). Working mixtures were diluted from the standard mixtures with methanol–water (1:1). Concentration of the working solutions of the internal standards dibenzepine and enalapril was 1 μg/ml in methanol–water (1:1).

2.3.2. Extraction procedure

Blood was spiked with working mixtures to obtain concentrations between 0.002 and 10 mg/l of free compound or salt, depending on the original reference compound. Extraction of the blood samples (1 ml) was carried out in two steps as described earlier [11]. (1) The basic internal standard (dibenzepine 20 μl, 1 μg/ml) was added to 1 ml of blood sample, which was made basic with Tris–buffer (pH 11) and extracted with butyl acetate. (2) The acidic internal standard (enalapril 20 μl, 1 μg/ml) and NaCl were added to the rest of the blood, which was then made acidic with phosphate buffer and phosphoric acid, and extracted with dichloromethane–isopropanol (95:5). The extracts were combined and analyzed by LC–MS–MS.

2.3.3. Liquid chromatography

LC separation was performed as described earlier [11]. The separation column was stabilized at 35 °C. The gradient involved acetonitrile and ammonium acetate buffer (10 m*M*, 0.1% formic acid, pH 3.2) as follows: CH_3CN 20–100% in 10 min and then 3 min isocratic. The total run time was 18 min, including 5 min equilibrium time at the beginning and 3 min cleaning of the column with 100% acetonitrile at the end. Total flow-rate through the column was 200 μl/min. Injection volume was 30 μl.

2.3.4. Mass spectrometry

Total eluent flow from the HPLC was directed into the turbo ionspray source without splitting. Needle voltage was 5.2 kV, and the nebulizer gas (air, 60 p.s.i.) and curtain gas (nitrogen, 40 p.s.i.) were set at 10 and 12 in the Sciex control software, respectively. The collision cell gas (nitrogen, 40 p.s.i.) in all MS–MS experiments was set at 2. The turbo ion spray heater temperature was set at 375 °C and the heater gas flow-rate at 7 l/min. The mass spectrometric analysis was performed in positive ion mode.

In addition to retention time, identification was based on the protonated molecular ion and one representative fragment ion. MRM was used for the detection of the total of 238 ion transitions. Collision energy was set at 20, 35, or 50 eV, depending on compound, selected on the basis of the most specific and intense fragment ion (Tables 1–4). Ion transitions were monitored with dwell times of 25 ms, and the total cycle time was 6 s.

2.3.5. Reference methods

LC–MS–MS results were compared with the accredited routine methods of our laboratory. In the capillary gas chromatographic (GC) method, the compounds were identified in the blood by a dual column system with retention index standards [12]. In the thin-layer chromatographic (TLC) and overpressured layer chromatographic (OPLC) methods, identification of compounds in urine or liver were based on their corrected R_f values, in situ UV spectra, and color reactions [13,14]. If the results obtained by the MRM screening and the routine methods were in disagreement, GC–MS served as a confirmation technique. GC–MS analysis was carried out in the selected ion mode (SIM), and identification of compounds was based on the retention time and two to four most abundant ions.

3. Results and discussion

The most important characteristics of the LC–MS–MS screening with MRM are summarized in Tables 1–4. The LOD values were determined by spiking whole blood samples with an appropriate series of the working standard mixtures. Replicates were extracted and analyzed in another day to check the reproducibility. The limit of detection (LOD) was defined by five criteria: (1) M+1 and the fragment ion were found, (2) the retention time difference was ±0.3 min with retention times less than 3 min, and ±0.2 min with retention times higher than 3 min, (3) the signal/noise ratio was at least three, (4) at least four data points were measured across the chromatographic peak, (5) the peak

Table 1
Protonated molecules (M+1), monitored fragments, collision energies (CE), LC retention times (RT), and limits of detection (LOD) of the screened compounds; lowest values studied marked "≤"

Compound	M+1	Fragment	CE (eV)	RT (min)	LOD (mg/l)
Acebutolol	337.0	319.0	20	3.8	0.1
Acrivastine	349.2	278.0	20	5.7	≤0.02
Alprazolam	325.2	297.0	35	6.1	≤0.02
Alprazolam, 1-hydroxy-	309.0	281.0	35	6.6	≤0.02
Alprenolol	250.2	173.0	20	5.4	0.01
Amantadine	152.2	135.3	20	3.4	0.1
Amiloride	230.2	171.0	20	2.0	0.1
Aminophenazone	232.2	113.0	20	2.8	≤5
Aminophenazone, 4-methyl-	218.2	187.0	20	2.6	≤5
Amiodarone	646.0	100.3	35	10.2	0.05
Amitriptyline	278.0	233.0	20	6.6	≤0.02
Astemizol	459.2	218.0	35	5.8	≤0.02
Atenolol	267.2	225.0	20	1.7	0.30
Azacyclonol	268.2	250.0	20	5.1	0.02
Benzhexol	302.2	98.0	35	6.6	≤0.02
Benzoylecgonine	290.2	168.0	20	3.3	0.01
Betaxolol	308.2	116.3	35	5.5	0.01
Biperidine	312.2	98.0	35	6.2	≤0.02
Bisoprolol	326.2	116.0	20	5.0	≤0.02
Bromipheniramine	319.2	274.2	20	5.3	0.002
Bupivacaine	289.2	140.3	35	5.1	≤0.02
Buprenorphine	468.2	396.3	50	5.9	0.01
Buspirone	386.2	122.0	50	5.1	0.002
Caffeine	195.0	138.0	35	2.8	1
Carbamazepine	237.0	194.0	20	6.1	≤0.02
Carbamazepine, 10-hydroxy-	255.2	237.0	20	4.5	0.1
Carbinoxamine	291.2	202.0	20	5.1	0.002
Carisoprodol	261.2	176.0	20	6.7	≤5
Carvedilol	407.2	222.0	35	6.2	≤0.02
Celiprolol	380.2	251.0	35	4.3	0.05
Cetirizine	389.2	201.2	35	6.3	0.05
Chlorcyclizine	201.0	166.0	20	6.6	≤0.02
Chlordiazepoxide	300.2	282.0	35	5.7	≤0.02
Chlormezanone	274.2	209.0	20	5.8	≤5
Chloroquine	320.0	247.0	35	2.7	0.02
Chlorpheniramine	275.2	230.2	20	5.1	0.002
Chlorpromazine	319.0	246.0	35	7.0	0.02
Chlorpropamide	277.0	192.0	20	6.7	≤5
Chlorprothixene	316.0	231.0	35	7.0	≤0.02
Cinnarizine	369.2	167.0	20	7.9	≤0.02
Citalopram	325.0	109.0	35	5.7	≤0.02
Citalopram, desmethyl-	311.2	262.0	20	5.5	≤0.02
Clemastine	344.2	215.2	20	7.7	0.02
Clobazam	301.2	259.0	35	7.3	≤0.02
Clobazam, nor-	287.0	245.0	35	5.8	0.1
Clobutinol	256.2	238.0	20	5.3	0.02
Clomethiazol	162.0	113.0	35	6.2	0.5
Clomipramine	315.0	86.0	35	7.1	≤0.02
Clomipramine, desmethyl-	301.2	270.3	20	6.9	0.02
Clonazepam	316.0	270.0	35	6.6	≤0.02
Clonazepam, 7-amino-	286.2	222.0	35	4.4	0.02
Clonidine	230.0	213.0	35	2.8	0.1
Clozapine	327.2	270.3	35	5.6	≤0.02
Cocaine	304.2	182.0	35	4.6	≤0.02
Codeine	300.2	215.0	35	2.5	0.1
Coumatetralyl	293.0	175.0	35	8.4	0.05
Cyclizine	267.4	167.2	20	5.8	≤0.02
Dextropropoxyphen	340.0	266.0	20	6.6	0.05
Demoxepam	287.0	269.0	35	5.8	0.02
Dextrometorphan	272.2	215.0	35	5.5	≤0.02

Table 2
Protonated molecules (M+1), monitored fragments, collision energies (CE), LC retention times (RT), and limits of detection (LOD) of the screened compounds; lowest values studied marked "≤"

Compound	M+1	Fragment	CE (eV)	RT (min)	LOD (mg/l)
Diazepam	285.0	222.0	35	8.1	0.02
Diazepam, desmet-	271.0	208.0	35	7.2	0.05
Diltiazem	415.0	178.0	35	5.8	≤0.02
Diphenhydramine	256.0	167.2	20	5.7	≤0.02
Dipyridamole	505.2	429.3	50	5.4	0.005
Disopyramine	340.2	239.0	20	4.4	≤0.02
Dixyrazine	428.2	229.0	35	6.8	0.005
Doxapram	379.2	292.3	35	4.8	≤0.02
Doxepine	280.2	235.0	20	5.9	≤0.02
Ebastine	470.2	203.0	35	9.6	0.005
Embutramide	294.2	208.0	20	6.7	0.005
Ergotamine	582.2	564.3	20	5.5	0.005
Ethenzamide	166.2	149.0	20	5.0	0.05
Ethylmorphine	314.2	229.0	35	3.2	0.05
Ethylparathion	292.0	236.0	20	9.7	≤5
Etodroxizine	419.2	201.0	35	6.4	≤0.02
Felodipine	384.2	338.2	20	9.6	0.02
Fenazepam	351.0	206.0	50	7.5	≤0.02
Fenfluramine	232.0	159.0	35	5.3	≤0.02
Fenkamfamine	216.4	171.0	20	5.1	≤0.02
Fentanyl	337.2	188.3	35	5.5	≤0.02
Fexofenadine	502.2	466.2	35	6.3	≤0.02
Flecainide	415.2	398.3	35	5.9	≤0.02
Fluconazole	307.2	238.0	20	4.0	0.1
Flumazenil	304.2	258.0	20	5.2	≤0.02
Flunitrazepam	314.2	268.3	35	7.1	0.002
Flunitrazepam, desmethyl-	300.2	254.0	35	6.4	0.02
Fluoxetine	310.2	148.0	20	6.8	0.1
Flupentixol	435.2	390.3	35	7.5	0.18
Fluvoxamine	319.2	259.0	20	6.3	0.02
Glibenclamide	494.0	369.0	20	8.5	≤0.02
Glipizide	446.2	321.0	20	6.8	≤0.05
Haloperidol	376.0	165.0	35	6.1	≤0.02
Histapyrrodine	281.2	210.3	20	6.3	0.02
Hydrocodone	300.2	199.0	35	3.0	0.05
Hydroxychloroquine	336.2	247.0	35	2.4	≤0.3
Hydroxyzine	375.2	201.0	35	6.3	≤0.02
Imipramine	281.2	208.0	35	6.4	0.05
Indomethacine	358.0	138.8	20	8.6	0.05
Isoniazide	138.2	121.0	20	2.2	3
Isradipine	372.4	340.0	20	8.6	0.05
Ketamine	238.2	220.0	20	3.6	≤0.05
Ketobemidone	248.2	190.3	35	3.3	≤0.05
Ketoprofen	255.0	209.0	35	7.3	0.1
Ketorolac	256.2	105.0	20	6.2	0.05
Labetalol	329.2	311.0	20	4.9	0.05
Lamotrigine	256.0	211.0	35	4.0	0.1
Levocabastine	421.2	375.0	35	5.8	0.01
Levomepromazine	329.0	242.0	35	6.5	0.02
Lidocaine	235.2	86.0	20	3.7	≤0.05
Loratadine	383.0	337.0	35	9.3	0.002
Lorazepam	321.2	303.0	20	6.6	0.02
Lormetazepam	335.0	289.0	35	7.4	≤0.02
LSD	324.2	223.0	35	4.7	≤0.02
Malathion	331.2	127.0	20	8.9	10
Maprotiline	278.2	250.0	20	6.4	≤0.02
MDMA	194.2	163.0	20	3.3	0.02
Meclozine	391.2	201.0	20	8.5	≤0.02
Medazepam	271.2	207.0	35	6.3	≤0.02
Meloxycam	352.0	115.0	35	7.1	0.01

Table 3
Protonated molecules (M+1), monitored fragments, collision energies (CE), LC retention times (RT), and limits of detection (LOD) of the screened compounds; lowest values studied marked ''≤''

Compound	M+1	Fragment	CE (eV)	RT (min)	LOD (mg/l)
Melperone	264.0	165.0	35	5.0	≤0.02
Mepivacaine	247.2	98.0	20	3.7	≤0.02
Meprobamate	241.2	139.0	20	4.9	0.1
Mesoridazine	387.2	372.3	35	5.4	≤0.02
Metamphetamine	150.2	91.0	20	3.3	0.05
Methadone	310.2	265.3	20	6.7	≤0.02
Methadone, nor-	296.2	251.0	20	6.3	0.05
Methylparathion	264.2	232.0	20	8.6	10
Methylphenidate	234.2	84.0	35	4.2	≤0.02
Metoclopramide	300.2	227.0	20	3.8	≤0.02
Metoprolol	268.2	191.0	20	4.1	0.02
Metronidazol	172.2	128.0	20	2.6	1
Mexiletine	180.2	58.0	20	4.4	0.05
Mianserine	265.2	208.0	20	5.7	≤0.02
Mianserine, desmethyl-	251.2	208.0	20	5.5	0.01
Midazolam	326.0	291.0	35	5.9	≤0.02
Midazolam, 1-hydroxy-	342.2	324.0	35	6.2	≤0.02
Mirtazapine	266.2	195.0	35	4.4	≤0.02
Mizolastine	433.2	308.4	20	5.5	0.01
Moclobemide	269.2	182.0	20	3.7	0.05
Molindone	277.2	100.0	35	4.0	≤0.02
Morphine	286.0	201.0	35	2.0	0.1
Morphine, 6-monoacetyl-	328.2	211.0	35	2.7	0.1
Nicotine	163.2	132.0	20	2.2	0.05
Nifedipine	347.2	254.0	20	7.5	0.02
Nikethamide	179.2	108.0	20	3.6	≤0.02
Nitrazepam	282.2	236.0	35	6.5	≤0.02
Nitrazepam, 7-amino-	252.2	121.0	35	3.5	≤5
Nizatidine	332.2	286.0	20	1.7	1
Nomifensine	239.2	196.0	20	4.6	≤0.02
Nortriptyline	264.2	233.3	20	6.4	≤0.02
Norverapamil	441.2	165.0	35	6.2	1
Noscapine	414.2	220.0	35	5.0	≤0.02
Olanzapine	313.2	256.0	35	3.0	0.05
Ondansetrone	294.2	170.0	35	4.6	≤0.02
Orphenadrine	270.4	181.2	20	6.1	≤0.02
Oxazepam	287.0	269.0	20	6.3	≤0.02
Oxcarbazepine	253.2	236.0	20	5.3	0.02
Oxprenolol	266.2	225.3	20	4.7	0.02
Oxycone	316.2	298.3	20	2.8	0.05
Papaverine	340.2	202.0	35	4.8	≤0.02
Paracetamol	152.0	110.0	20	2.5	≤5
Paroxetine	330.2	192.0	35	6.2	0.02
Pemoline	177.0	106.2	20	3.3	0.05
Pentazocine	286.2	218.3	35	5.0	≤0.02
Pentifylline	265.2	138.0	35	7.3	≤5
Pentoxyverine	334.2	100.0	35	6.6	≤0.02
Perphenazine	404.2	171.3	35	6.9	0.002
Pethidine	248.2	220.3	35	4.7	≤0.02
Phenazone	189.2	147.0	35	3.9	0.05
Phencyclidine	244.2	159.0	20	5.3	0.05
Pheniramine	241.2	196.0	20	4.1	0.02
Phenylbutazone	309.2	160.3	35	9.0	≤5
Phenylpropanolamine	152.2	134.2	20	2.5	0.3
Phenytoin	253.2	182.3	20	6.1	0.05
Pindolol	249.2	172.0	20	3.3	0.05
Piroxycam	332.0	164.0	20	6.6	0.02
Pitofenone	368.2	112.0	35	5.4	≤0.02
Pizotifen	296.2	96.0	35	6.5	≤0.02
Practolol	267.2	225.0	20	1.8	0.1

Table 4
Protonated molecules (M+1), monitored fragments, collision energies (CE), LC retention times (RT), and limits of detection (LOD) of the screened compounds; lowest values studied marked ''≤''

Compound	M+1	Fragment	CE (eV)	RT (min)	LOD (mg/l)
Prazosin	384.0	247.0	35	4.1	0.05
Prilocaine	221.2	86.0	20	3.8	≤0.02
Primidone	219.2	162.3	20	4.0	≤5
Procainamide	236.0	163.0	20	2.2	0.05
Prochlorperazine	374.2	141.0	35	7.5	0.02
Promazine	285.0	86.0	20	6.2	≤0.02
Prometazin	285.2	240.0	20	6.0	0.05
Propafenone	342.2	324.0	20	6.3	≤0.02
Propranolol	260.2	155.0	35	5.4	0.02
Propyphenazone	231.2	189.0	20	6.6	0.50
Pseudoephedrine	166.2	148.0	20	2.6	1
Quinine	325.2	307.0	35	4.2	0.02
Ranitidine	315.2	176.0	20	1.8	0.1
Risperidone	411.2	191.0	35	4.9	≤0.02
Rocurone	529.4	487.2	35	3.8	0.1
Ropivacaine	275.2	126.0	35	4.6	≤0.02
Salicylamide	138.2	121.2	20	4.2	≤5
Selegiline	188.2	119.2	20	4.1	0.05
Sertindol	441.2	113.0	35	7.2	≤0.02
Sertraline	306.0	275.2	20	6.8	0.02
Sulindac	357.0	233.0	35	6.5	0.02
Simazine	202.3	104.0	35	6.0	0.1
Sincocaine	344.2	271.3	35	6.5	≤0.02
Sisapride	466.2	184.0	35	5.9	≤0.02
Sotalol	273.2	255.0	20	2.1	0.1
Strychnine	335.2	184.0	50	5.3	0.05
Sulpride	342.2	214.0	35	1.9	0.1
Sulthiame	291.0	185.0	35	4.1	0.05
Temazepam	301.0	255.0	35	7.2	≤0.02
Terbutaline	226.2	152.0	20	2.3	0.1
Terfenadine	472.2	436.4	35	8.1	0.002
Terodiline	282.2	226.2	20	6.7	≤0.02
Tetracaine	265.2	176.0	20	5.7	≤0.02
Tetrahydrocannabinol	315.2	193.2	20	12.3	0.05
Tetryzoline	201.2	131.0	35	3.6	0.1
Theobromine	181.0	138.0	20	2.3	≤5
Theophylline	181.2	124.2	20	2.4	≤5
Thioridazine	371.0	126.0	35	7.5	0.02
Thioridazine, 5-sulfoxy-	387.2	244.0	50	5.3	0.02
Timolol	317.2	261.0	20	3.8	0.05
Tiotixene	444.2	335.2	35	6.7	0.02
Tolbutamide	271.2	155.0	20	7.1	≤5
Toremifen	406.2	72.2	35	8.7	0.02
Tramadol	264.0	58.0	20	4.2	0.02
Trazodone	372.0	176.0	35	5.2	≤0.02
Triamteren	253.8	237.0	35	3.2	0.1
Triazolam	343.0	308.0	35	6.7	0.002
Triazolam, 1-hydroxy-	359.0	250.0	50	6.1	0.02
Trimeprazine	299.2	100.0	35	6.4	≤0.02
Trimethoprim	291.2	230.0	35	3.1	0.05
Trimipramine	295.2	100.0	20	6.7	≤0.02
Trimipramine, desmethyl-	281.4	86.0	20	6.7	≤0.02
Venlafaxine	278.2	260.3	20	4.9	0.02
Verapamil	455.2	165.0	35	6.5	≤0.02
Warfarin	309.2	251.0	20	7.9	≤0.02
Yohimbine	355.2	144.0	35	4.5	≤0.02
Zolpidem	308.2	235.3	35	4.7	≤0.02
Zopiclone	389.0	245.0	20	4.0	0.1

areas for the internal standards were above the minimum limit, showing that both the basic and the acidic extractions had been successful. LOD was ≤0.05 mg/l for 80% of the compounds. However, because subtherapeutic concentrations were not studied, for some compounds, LOD may thus be even lower than the values reported in Tables 1–4.

Ion suppression, caused by matrix, can effect the LOD values. Matrix effects were tested earlier by the present authors by spiking antihistamines to autopsy blood samples [11]. The average relative standard deviation within samples was 11%, while between samples it was 21%, showing that the matrix effect for these compounds was approximately 10%. Müller et al. [15] have recently studied the ion suppression effect after different extraction procedures of serum, and concluded that it is critical only in the LC-front peak but not during the rest of the gradient.

Tables 1–4 shows that the LOD obtained for a majority of the 238 compounds was low enough for forensic and clinical toxicology. For only seven compounds was LOD clearly higher than the therapeutic concentration range (C_{ther}). C_{ther} and LOD for those compounds were as follows: amiloride ≈0.04 and 0.2 mg/l, flupenthixol 0.001–0.015 and 0.18 mg/l, isradipine 0.0005–0.002 and 0.05 mg/l, clemastine <0.002 and 0.02 mg/l, clonidine 0.0003–0.0015 and 0.1 mg/l, terbutaline 0.001–0.006 and 0.1 mg/l, and levocabastine 0.01 and ad 0.002 mg/l, respectively.

For more compact reporting of screening results for these 238 compounds, we modified the PE Sciex TurboQuan 1.0 program and divided the results table into two pages. In the results tables (Fig. 1), all compounds searched for were listed by name, and only in cases of a positive finding was the name followed by the peak area. All compounds and their integration parameters can be viewed and printed from a separate window (Fig. 2).

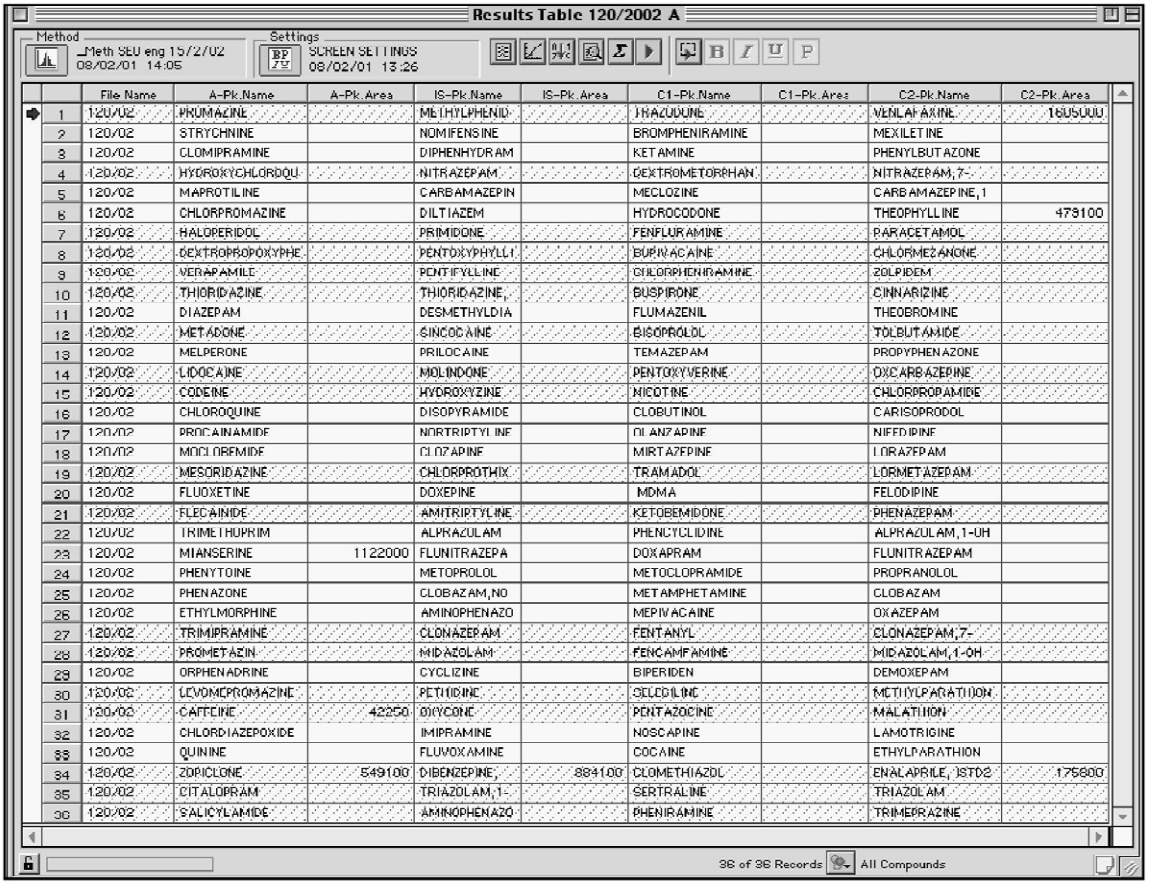
An example of the report for an autopsy blood sample (120/02) is Fig. 1. Peak areas for the internal standards (dibenzepin and enalapril) fell within the acceptable range. Drugs found in the MRM screening as well as in the reference GC analysis were zopiclone 0.5 mg/l, mianserine 0.2 mg/l, theophylline 7.5 mg/l, and caffeine <1 mg/l. Desmethylmianserine, venlafaxine, and risperidone were detected only in the MRM screening and were verified by GC–MS-SIM.

As shown in Table 5, method performance was tested by analyzing 71 blood samples taken at autopsy and comparing the results with those obtained with our reference methods (see Section 2). The samples consisted of a randomly selected series of 20 and 12 successive samples, and in addition, 39 samples were selected based on the results obtained by the reference methods to broaden the selection of compounds. Findings by the reference methods were regarded as correct.

Of the findings by MRM screening, 256 (92%) were consistent with those obtained by GC in blood or TLC/OPLC in urine or liver. Sixty-eight different compounds were identified by MRM. In 55 autopsy cases of the 71 studied, 76 findings were only by MRM. Metabolites at concentrations lower than the LOD of the reference methods explain 28 of these findings. The other cases were re-analyzed by GC–MS-SIM to confirm the results, and confirmation analysis showed that 30 of these findings were true-positives and 18 apparently false-positives. Some of the additional positive findings by MRM may be due to the fact that the selection of compounds included in our MRM library was not exactly the same as in the reference methods. In practical casework, all MRM screening results are always confirmed by another independent method and/or other sample material.

Interference from matrix was estimated by analyzing blank samples (bovine blood), which were found clean, and 12 autopsy samples that by reference methods were found negative or contained only caffeine. The only detected interference was the transition of 275.2→126.0 (ropivacaine), which was present at times also in other samples at low intensities. Therefore, the peak detection threshold was increased for this compound.

As can be seen in Table 5, caffeine was the most common finding in autopsy blood samples, but because of its poor peak shape, it was also often missed by MRM at subtherapeutic levels. Two metabolites of caffeine, theobromine and theophylline, were in some cases detected instead. Because the C_{ther} for caffeine is 8–15 mg/l, missing caffeine in these cases was not of toxicological importance. By MRM, the other missed findings were oxazepam



Results Table 120/2002 A

Method: Meth SEU eng 15/2/02 08/02/01 14:05 — Settings: SCREEN SETTINGS 08/02/01 13:26

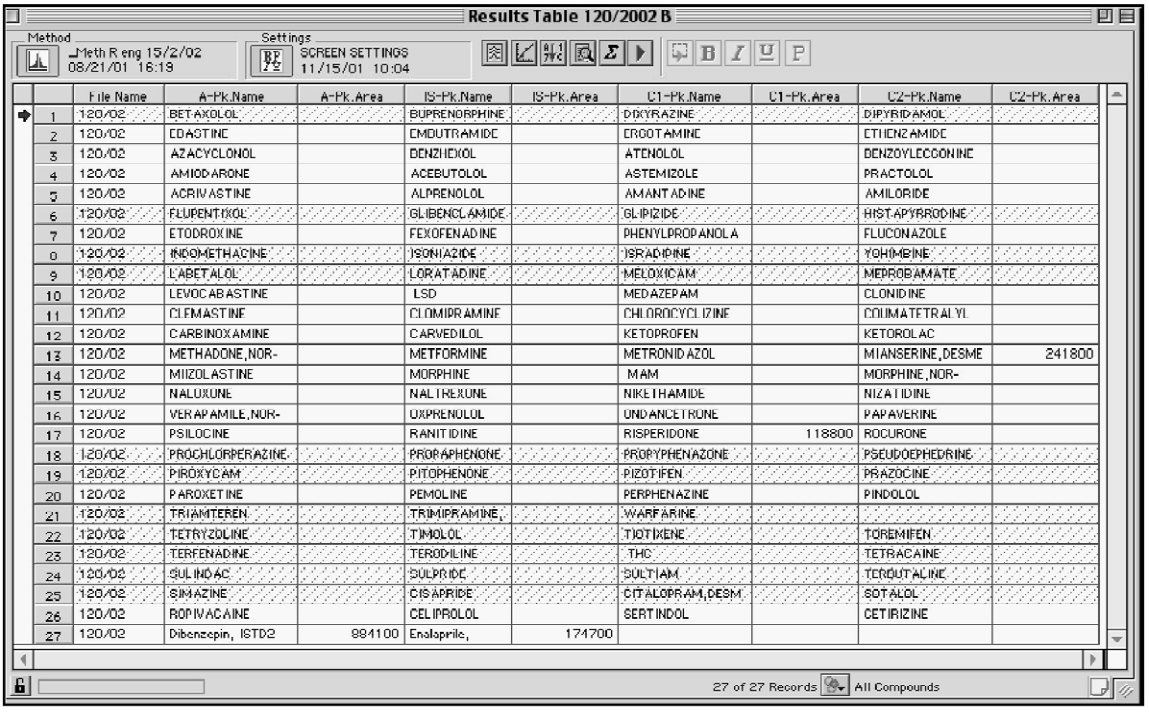
	File Name	A-Pk Name	A-Pk Area	IS-Pk Name	IS-Pk Area	C1-Pk Name	C1-Pk Area	C2-Pk Name	C2-Pk Area
1	120/02	PROMAZINE		METHYLPHENID		TRAZODONE		VENLAFAXINE	1605000
2	120/02	STRYCHNINE		NOMIFENSINE		BROMPHENIRAMINE		MEXILETINE	
3	120/02	CLOMIPRAMINE		DIPHENHYDRAM		KETAMINE		PHENYLBUTAZONE	
4	120/02	HYDROXYCHLOROQU		NITRAZEPAM		DEXTROMETORPHAN		NITRAZEPAM,7-	
5	120/02	MAPROTILINE		CARBAMAZEPIN		MECLOZINE		CARBAMAZEPINE,1	
6	120/02	CHLORPROMAZINE		DILTIAZEM		HYDROCODONE		THEOPHYLLINE	479100
7	120/02	HALOPERIDOL		PRIMIDONE		FENFLURAMINE		PARACETAMOL	
8	120/02	DEXTROPROPOXYPHE		PENTOXYPHYLLI		BUPIVACAINE		CHLORMEZANONE	
9	120/02	VERAPAMILE		PENTIFYLLINE		CHLORPHENIRAMINE		ZOLPIDEM	
10	120/02	THIORIDAZINE		THIORIDAZINE,		BUSPIRONE		CINNARIZINE	
11	120/02	DIAZEPAM		DESMETHYLDIA		FLUMAZENIL		THEOBROMINE	
12	120/02	METADONE		SINCOCAINE		BISOPROLOL		TOLBUTAMIDE	
13	120/02	MELPERONE		PRILOCAINE		TEMAZEPAM		PROPYPHENAZONE	
14	120/02	LIDOCAINE		MOLINDONE		PENTOXYVERINE		OXCARBAZEPINE	
15	120/02	CODEINE		HYDROXYZINE		NICOTINE		CHLORPROPAMIDE	
16	120/02	CHLOROQUINE		DISOPYRAMIDE		CLOBUTINOL		CARISOPRODOL	
17	120/02	PROCAINAMIDE		NORTRIPTYLINE		OLANZAPINE		NIFEDIPINE	
18	120/02	MOCLOBEMIDE		CLOZAPINE		MIRTAZEPINE		LORAZEPAM	
19	120/02	MESORIDAZINE		CHLORPROTHIX		TRAMADOL		LORMETAZEPAM	
20	120/02	FLUOXETINE		DOXEPINE		MDMA		FELODIPINE	
21	120/02	FLECAINIDE		AMITRIPTYLINE		KETOBEMIDONE		PHENAZEPAM	
22	120/02	TRIMETHOPRIM		ALPRAZOLAM		PHENCYCLIDINE		ALPRAZOLAM,1-OH	
23	120/02	MIANSERINE	1122000	FLUNITRAZEPA		DOXAPRAM		FLUNITRAZEPAM	
24	120/02	PHENYTOINE		METOPROLOL		METOCLOPRAMIDE		PROPRANOLOL	
25	120/02	PHENAZONE		CLOBAZAM,NO		METAMPHETAMINE		CLOBAZAM	
26	120/02	ETHYLMORPHINE		AMINOPHENAZO		MEPIVACAINE		OXAZEPAM	
27	120/02	TRIMIPRAMINE		CLONAZEPAM		FENTANYL		CLONAZEPAM,7-	
28	120/02	PROMETAZIN		MIDAZOLAM		FENCAMFAMINE		MIDAZOLAM,1-OH	
29	120/02	ORPHENADRINE		CYCLIZINE		BIPERIDEN		DEMOXEPAM	
30	120/02	LEVOMEPROMAZINE		PETIDINE		SELEGILINE		METHYLPARATHION	
31	120/02	CAFFEINE	42250	OXYCONE		PENTAZOCINE		MALATHION	
32	120/02	CHLORDIAZEPOXIDE		IMIPRAMINE		NOSCAPINE		LAMOTRIGINE	
33	120/02	QUININE		FLUVOXAMINE		COCAINE		ETHYLPARATHION	
34	120/02	ZOPICLONE	549100	DIBENZEPINE,	884100	CLOMETHIAZOL		ENALAPRILE, ISTD2	175800
35	120/02	CITALOPRAM		TRIAZOLAM,1-		SERTRALINE		TRIAZOLAM	
36	120/02	SALICYLAMIDE		AMINOPHENAZO		PHENIRAMINE		TRIMEPRAZINE	

36 of 36 Records — All Compounds

Fig. 1. Results table for sample 120/2002 (page 1/2). Positive findings and internal standards are indicated by their corresponding peak areas.

at levels 0.1 and 0.03 mg/l (C_{ther} 0.1–1.4 mg/l), alprazolam at 0.05 and 0.03 mg/l (C_{ther} 0.01–0.02 mg/l), zopiclone at 0.2 mg/l (C_{ther} 0.01–0.05 mg/l), levomepromazine at 0.2 mg/l (C_{ther} 0.05–0.14 mg/l), nicotine twice at 0.1 mg/l (C_{ther} 0.01–0.04 mg/l), olanzapine at 0.2 mg/l (C_{ther} ad 0.2 mg/l), and theophylline at 5.5 mg/l (C_{ther} 8–20 mg/l).

In a multiple-compound MRM analysis, the possibility of cross-talk has to be considered. Cross-talk may take place if two compounds coeluting and having the same fragment ions are monitored in two successive transitions. The extent of this problem depends on instrument speed: the fragment ions should pass through the collision cell fast enough to exit the cell before the same fragments of the next compound come in. One way to fix this problem is to use a pause time between the transitions, but this causes ''dead time'', making the scan cycle time significantly longer, which leads to fewer data points during a chromatographic peak. Our selection of compounds included only four pairs of compounds for which cross-talk really was an issue (same RT and the same fragment ion): atenolol and practolol (1.7 min; 267→225 and 267→225), fenfluramine and phencyclidine (5.3 min; 232→159 and 244→159), cetirizine and hydroxyzine (6.3 min; 389→201 and 375→201), and nortriptyline and sulindac (6.4 min; 264→233 and 357→233). Atenolol and practolol have the same parent and fragment ions, and the same retention time by the LC system used, but they can be separated with a very slow gradient. For all other analytes in our applica-



Results Table 120/2002 B

Method: Meth R eng 15/2/02 08/21/01 16:19 — Settings: SCREEN SETTINGS 11/15/01 10:04

	File Name	A-Pk.Name	A-Pk.Area	IS-Pk.Name	IS-Pk.Area	C1-Pk.Name	C1-Pk.Area	C2-Pk.Name	C2-Pk.Area
1	120/02	BETAXOLOL		BUPRENORPHINE		DIOXYRAZINE		DIPYRIDAMOL	
2	120/02	EBASTINE		EMDUTRAMIDE		ERGOTAMINE		ETHENZAMIDE	
3	120/02	AZACYCLONOL		BENZHEXOL		ATENOLOL		BENZOYLECGONINE	
4	120/02	AMIODARONE		ACEBUTOLOL		ASTEMIZOLE		PRACTOLOL	
5	120/02	ACRIVASTINE		ALPRENOLOL		AMANTADINE		AMILORIDE	
6	120/02	FLUPENTIXOL		GLIBENCLAMIDE		GLIPIZIDE		HISTAPYRRODINE	
7	120/02	ETODROXINE		FEXOFENADINE		PHENYLPROPANOLA		FLUCONAZOLE	
8	120/02	INDOMETHACINE		ISONIAZIDE		ISRADIPINE		YOHIMBINE	
9	120/02	LABETALOL		LORATADINE		MELOXICAM		MEPROBAMATE	
10	120/02	LEVOCABASTINE		LSD		MEDAZEPAM		CLONIDINE	
11	120/02	CLEMASTINE		CLOMIPRAMINE		CHLORDIAZEPOXIDE		COUMATETRALYL	
12	120/02	CARBINOXAMINE		CARVEDILOL		KETOPROFEN		KETOROLAC	
13	120/02	METHADONE,NOR-		METFORMINE		METRONIDAZOL		MIANSERINE,DESME	241800
14	120/02	MIZOLASTINE		MORPHINE		MAM		MORPHINE,NOR-	
15	120/02	NALOXONE		NALTREXONE		NIKETHAMIDE		NIZATIDINE	
16	120/02	VERAPAMILE,NOR-		OXPRENOLOL		ONDANCETRONE		PAPAVERINE	
17	120/02	PSILOCINE		RANITIDINE		RISPERIDONE	118800	ROCURONE	
18	120/02	PROCHLORPERAZINE		PROPAPHENONE		PROPYPHENAZONE		PSEUDOEPHEDRINE	
19	120/02	PIROXYCAM		PITOPHENONE		PIZOTIFEN		PRAZOCINE	
20	120/02	PAROXETINE		PEMOLINE		PERPHENAZINE		PINDOLOL	
21	120/02	TRIAMTEREN		TRIMIPRAMINE,		WARFARINE			
22	120/02	TETRYZOLINE		TIMOLOL		TIOTIXENE		TOREMIFEN	
23	120/02	TERFENADINE		TERODILINE		THC		TETRACAINE	
24	120/02	SULINDAC		SULPRIDE		SULTIAM		TERBUTALINE	
25	120/02	SIMAZINE		CISAPRIDE		CITALOPRAM,DESM		SOTALOL	
26	120/02	ROPIVACAINE		CELIPROLOL		SERTINDOL		CETIRIZINE	
27	120/02	Dibenzepin, ISTD2	884100	Enalaprile,	174700				

27 of 27 Records — All Compounds

Fig. 1. (*continued*)

tion, the order of MRM transitions was arranged so that the time between the same fragment ion originating from different parent ions was as long as possible, at least 200 ms (with eight other compounds between parents, 25 ms/transition). Additionally, a difference of at least 5 u was used between consecutive fragment ions, and with this procedure we noticed no cross-talk effects. This was in agreement with findings of Tong et al. [9], who studied the effect of mass differences on cross-talk.

Dwell time, which is the time used for monitoring each ion transition, has a significant effect on detection limits. Generally, dwell time should be maximized to improve signal-to-noise ratio. With our instrument, however, dwell times longer than 100 ms did not improve the ratio further (Fig. 3), but instead increased the threshold. While processing as many as 238 compounds, the upper limit for dwell time was restricted by the number of data points across the chromatographic peak: for qualitative purposes, four data points are enough to pick up the peak from the background reliably, whereas for quantitation purposes, 10–15 data points are required for good peak shape and reproducible integration. This resulted in a compromise value of 25 ms dwell time for all 238 compounds and a total cycle time of 6 s, which means that the chromatographic peak width must be at least 24 s, and therefore the chromatographic run must not be too fast, producing sharp peaks which will be undetected. We showed that at the LOD of the studied compounds, four to six data points per peak were obtainable in a 10-min chromatographic run.

Splitting the chromatographic run into time windows according to compound retention times is also possible, so that more data points per peak can be achieved because the number of transitions in each time window will be smaller. Fillion et al. [16] have used this approach with gas chromatography, in which accuracy and reproducibility of retention times is better than in liquid chromatography. In our selection of compounds, differences in retention times between successively eluting compounds were very small, less than 0.1 min for the first 217 compounds. Consequently, even small fluctuations in retention times will cause a peak to slide outside its

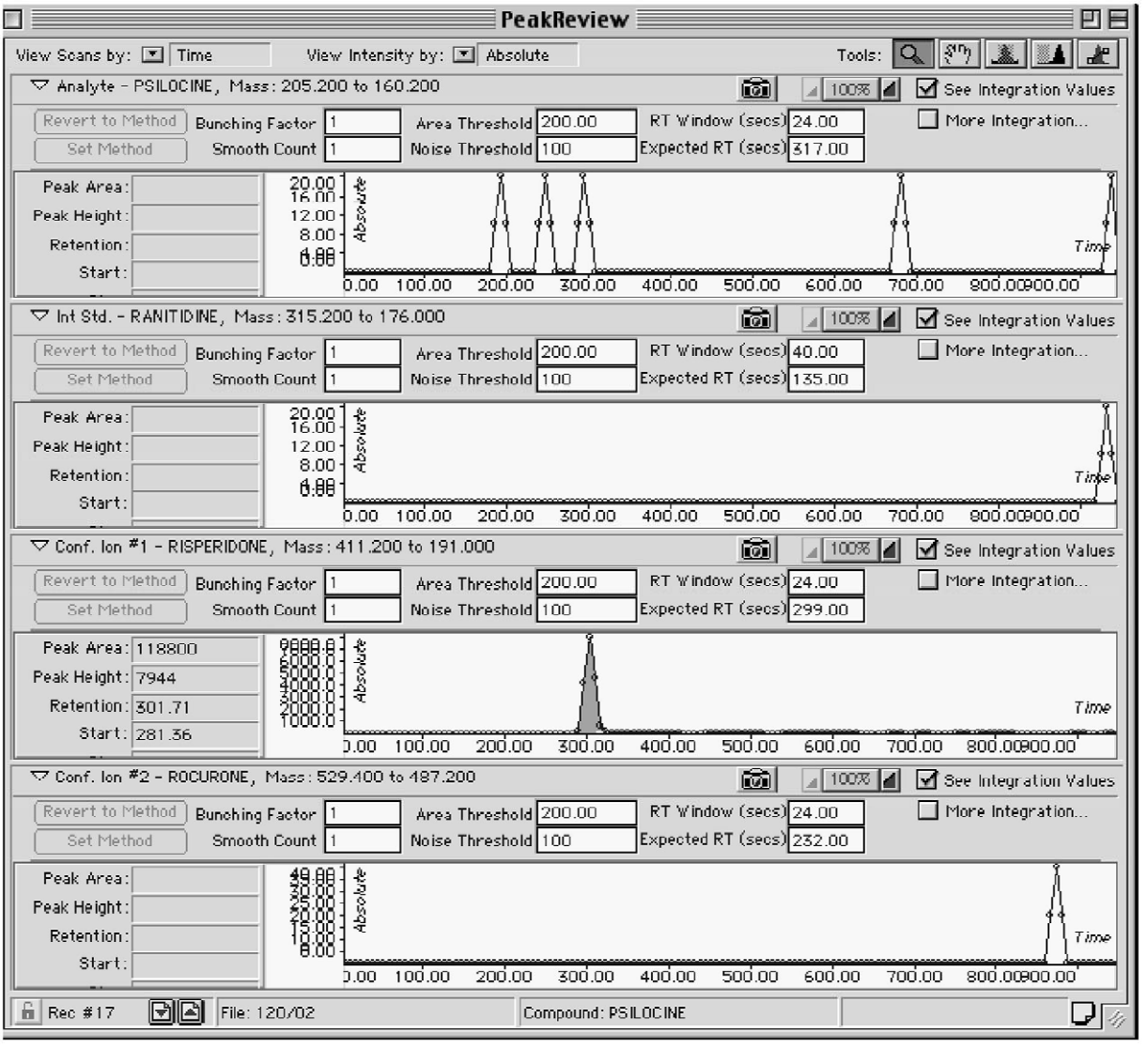


Fig. 2. Example of the peak review window (sample 120/2002). Names, mass transitions, integration parameters, and MRM-chromatograms of four compounds can be examined at the same time in one review window. Peaks fitting the criteria for positive identification are darkened.

time window; all compounds close to the time window borders should therefore be monitored in two successive windows. Because this approach would have made this method less robust and much more complicated, we chose not to apply it.

An important advantage of MRM is that coeluting compounds do not interfere, as with in-source CID, where coeluting compounds are fragmented simultaneously in the ion source and therefore interfere with identification. Another benefit is its high sensitivity. In LC–MS with in-source CID [2–4] or LC–MS–MS with DDE [6,7], the instrument is operated in scanning mode during the screening step, whereas in MRM, better sensitivity is achieved because of longer dwell times per ion.

The number of compounds in the present MRM

Table 5
Comparison of findings by LC–MS–MS and the GC, TLC or OPLC reference methods: results for 71 autopsy cases

Compound	Number of findings		Compound	Number of findings	
	Ref. methods	LC–MS–MS		Ref. methods	LC–MS–MS
Caffeine	40	31	Theophylline	2	1
Desmethyldiazepam	22	22	Thioridazine, 5-sulfoxy-	2	2
Oxazepam	16	14	Venlafaxine	2	2
Temazepam	16	19	Verapamil	2	2
Diazepam	14	14	Warfarin	2	2
Citalopram	13	13	Acebutolol	1	1
Paracetamol	11	11	Amiodarone	1	1
Zopiclone	8	7	Bupivacaine	1	1
Carbamazepine	7	7	Carbamazepine, OH-	1	1
Citalopram, desmethyl-	7	10	Chloroquine	1	1
Lidocaine	7	7	Clomethiazol	1	1
Chlordiazepoxide	6	6	Codeine	1	1
Nicotine	6	4	Dextromethorphane	1	1
Promazine	6	6	Dextropropoxyphene	1	1
Demoxepam	5	5	Fluoxetine	1	1
Tramadol	5	5	Hydroxyzine	1	1
Clozapine	4	4	Mesoridazine	1	1
Levomepromazine	4	3	Methadone	1	1
Mianserine	4	4	Metoclopramide	1	1
Mirtazapine	4	4	Metoprolol	1	1
Phenytoin	4	4	Moclobemide	1	1
Alprazolam	3	1	Oxycodone	1	1
Amitriptyline	3	3	Propranolol	1	1
Chlorprothixene	3	3	Quinine	1	1
Diltiazem	3	3	Risperidone	1	1
Doxepine	3	3	Ropivacaine	1	1
Orphenadrine	3	3	Theobromine	1	1
Thioridazine	3	3	Trazodone	1	1
Chlorpromazine	2	2	Midazolam	0	1
Fluconazole	2	2	Glibenclamide	0	1
Ketoprofen	2	2	Trimipramine	0	1
Lamotrigine	2	2	Glipizide	0	1
Melperone	2	2	Acrivastine	0	1
Olanzapine	2	1			

screening was not as high as in the methods using a scan mode for screening. Those types of methods are, however, also limited to the compounds included in their spectrum libraries, the largest published libraries ranging between 400 and 1000 drugs [2–4,11]. Screening simultaneously 238 MRM transitions is, however, more than in previously reported methods of this type, and the number of drugs can be increased still further by use of an LC–MS–MS instrument with higher scan speed. Simultaneous quantitation, as described with antihistamines [11], is possible with newer software (e.g., PE Sciex Analyst 1.3) and a faster instrument.

4. Conclusions

Our LC–MS–MS method utilizing MRM enabled us to screen for 238 therapeutic and illegal drugs in autopsy blood samples. The method was sufficiently selective and sensitive to detect the majority of compounds down to therapeutic concentration levels

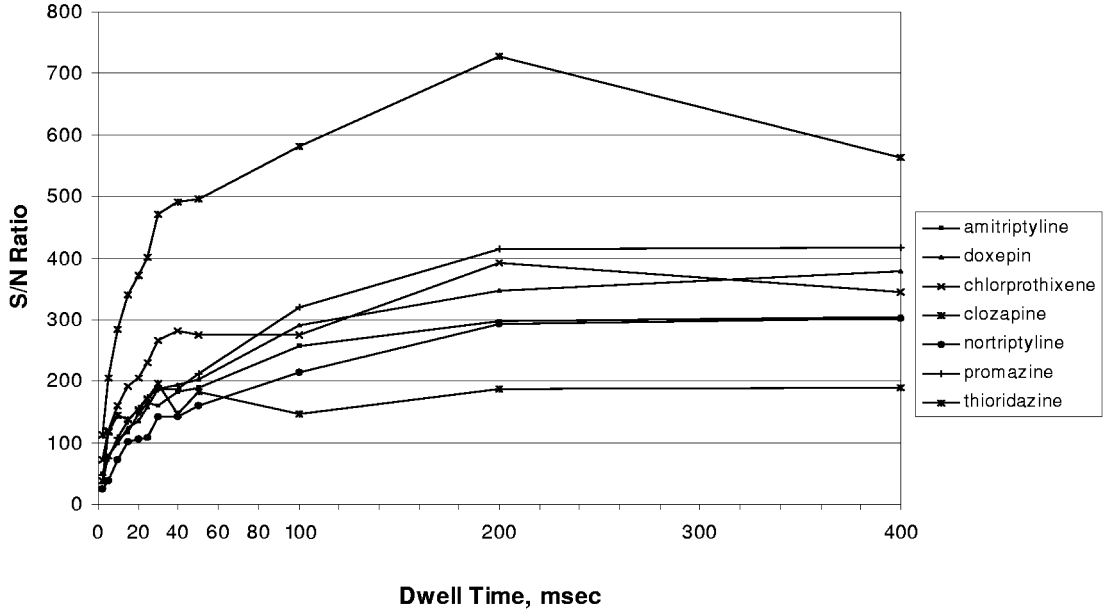


Fig. 3. The effect of dwell time on signal-to-noise ratio.

and is therefore feasible in forensic and clinical toxicology. Comparison with TLC, OPLC, GC, and GC–MS methods showed that MRM screening was reliable and generally more sensitive than the reference methods. A limitation of the present method was that, due to the relatively low scan speed of the instrument in use, only one fragment ion could be used for identification.

References

[1] P. Marquet, Ther. Drug Monit. 24 (2002) 123.
[2] P. Marquet, N. Venisse, E. Lacassie, G. Lachatre, Analusis 28 (2000) 925.
[3] F. Saint-Marcoux, G. Lachatre, P. Marquet, J.Am. Soc. Mass Spectrom. 14 (2003) 14.
[4] W. Weinmann, A. Wiedemann, B. Eppinger, M. Renz, M. Svoboda, J. Am. Soc. Mass Spectrom. 10 (1999) 1028.
[5] M. Rittner, F. Pragst, W.-R. Bork, J. Neumann, J. Anal. Toxicol. 25 (2001) 115.
[6] R.L. Fitzgerald, J.D. Rivera, D.A. Herold, Clin. Chem. 45 (1999) 1224.
[7] T.N. Decaestecker, K.M. Clauwaert, J.F. Van Bocxlaer, W.E. Lambert, E.G. Van den Eeckhout, C.H. Van Peteghem, A.P. De Leenheer, Rapid Commun. Mass Spectrom. 14 (2000) 1787.
[8] M. Gergov, B. Boucher, I. Ojanperä, E. Vuori, Rapid Commun. Mass Spectrom. 15 (2001) 521.
[9] X. Tong, I.E. Ita, J. Wang, J.V. Pivnichny, J. Pharm. Biomed. Anal. 20 (1999) 773.
[10] D. Thieme, J. Grosse, R. Lang, R.K. Mueller, A. Wahl, J. Chromatogr. B 757 (2001) 49.
[11] M. Gergov, N.J. Robson, I. Ojanperä, O.P. Heinonen, E. Vuori, Forensic Sci. Int. 121 (2001) 108–115.
[12] I. Rasanen, I. Ojanperä, J. Vartiovaara, E. Vuori, P. Sunila, J. High Resolut. Chromatogr. 19 (1996) 313.
[13] I. Ojanperä, in: Practical Thin-Layer Chromatography: a Multidisciplinary Approach, CRC Press, Boca Raton, FL, 1996.
[14] I. Ojanperä, K. Goebel, E. Vuori, J. Liq. Chrom. Technol. 22 (1999) 161.
[15] C. Müller, P. Schäfer, M. Störzel, S. Vogt, W. Weinmann, J Chromatogr. B 773 (2002) 47.
[16] J. Fillion, R. Hindle, M. Lacroix, J. Selwyn, J. AOAC Int. 78 (1995) 1252.