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Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS) method was developed and validated for simultaneous analysis of nine acidic pharmaceutical drugs (bezafibrate, clofibric acid, diclofenac, fenoprofen, gemfibrozil, ibuprofen, indomethacin, ketoprofen and naproxen) in sewage treatment plant (STP) effluents. The mean recoveries of the pharmaceuticals ranged from 58.9 to 91.5% in STP effluent, and the limits of detection of the analytes were 5–20 ng/ml. The method was applied to the quantitative analysis of acidic drugs in the effluents from three Canadian STPs, in which bezafibrate, diclofenac, fenoprofen, gemfibrozil, ibuprofen, indomethacin and naproxen were detected. © 2002 Published by Elsevier Science B.V.

Keywords: Tandem mass spectrometry; Electrospray ionization; Acidic drugs

1. Introduction

The fate of pharmaceutical and personal care products (PPCPs) is an emerging issue in environmental research [1–4]. It has been hypothesized that drugs released into the environment could have subtle effects on wildlife and humans [5,6], and risk assessments have predicted impacts of PPCPs on the environment [7,8]. There is a widespread consensus that PPCP contamination may require legislative intervention. Many PPCPs are highly bioactive, most are polar, and when present in the environment, they usually occur at part per billion or part per trillion concentrations. These factors present a number of analytical challenges. Sensitive and selective analytical procedures for PPCPs are necessary to determine the environmental distribution of these compounds.

Acidic pharmaceutical drugs are a major group of PPCPs that have been widely detected in the aquatic environment [5]. Bezafibrate and Naproxen were detected in German municipal sewage treatment plant (STP) effluents at concentrations up to 4.6 μg/l [9] and 0.4 μg/l [10], respectively. In a recent survey of acidic drugs in Canadian STP effluents, ibuprofen and naproxen were detected in most effluents, gemfibrozil and bezafibrate were observed in some effluents, and clofibric acid was not detected

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in any STP effluents [11]. These compounds are thermolabile and nonvolatile, and previously have been analyzed by GC–MS (or MS–MS) after derivatization [9–15], which makes the sample preparation laborious and time consuming, increases the possibility of contamination and errors, and may lead to degradation of labile compounds. Liquid chromatography–mass spectrometry (LC–MS) is an appropriate technique to analyze the polar and thermolabile compounds [13,16,17]. Recently, some acidic drugs were analyzed in aqueous samples using LC–MS and CE–MS [16,17]. LC–MS–MS is more suitable for environmental analysis because of its specificity and selectivity. The aim of this study is to develop an LC–MS–MS method for determining acidic drugs in aqueous samples. We used solid-phase extraction (SPE) and LC–ESI-MS–MS for analysis of underivatized acidic drugs, and validated the method by analysis of STP effluent samples. The compounds investigated are frequently detected pharmaceuticals, including lipid regulators and metabolites, analgesics, and anti-inflammatory or antiphlogistic agents.

2. Experimental

2.1. Reagents and standards

The acidic pharmaceutical standards, bezafibrate, clofibric acid, diclofenac, fenoprofen, gemfibrozil, ibuprofen, indomethacin, ketoprofen and naproxen were purchased from Sigma (St Louis, MO, USA). Table 1 shows the CAS registry numbers, chemical structures, and the use and origin of the pharmaceuticals studied. All of the standard compounds were powders, and were dissolved in an appropriate volume of methanol–water (1:1, v/v) to yield stock (100 μg/ml) and working solutions of each individual compound, which were stored in brown glass bottles at 4 °C, and brought to room temperature before use.

HPLC grade acetonitrile and methanol were supplied by Caledon Laboratories Ltd. (Georgetown, Ont., Canada), and HPLC grade water was supplied by EM Science Industries, Inc. (Gibbstown, NJ, USA). Ammonium acetate (98%) purchased from Sigma (St Louis, MO, USA) was used without further purification.

2.2. Sample preparation

Effluent samples were collected in solvent-washed glass bottles, which were rinsed with tap water and HPLC-grade water prior to sampling. Samples of final effluent were collected from two STPs (STP-1 and STP-2) in Whitby, Ont., on April 26, 2001 and one STP (STP-3) in Peterborough, Ont., on September 10, 2001, and they were extracted within 12 h of collection.

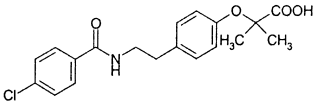
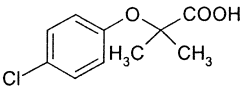
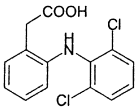
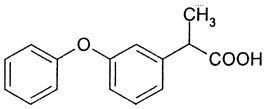
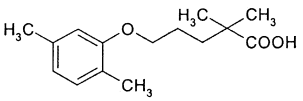
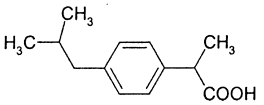
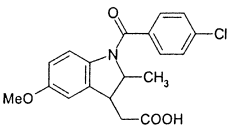
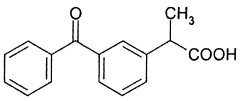
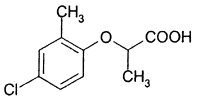
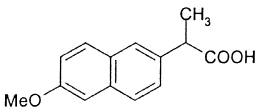
The SPE extraction of the acidic drugs was conducted as described by Metcalfe et al. [11]. Briefly, a 500-ml STP effluent was extracted with SPE cartridges consisting of 0.5 g of LiChrolut 100 RP-18 (40–63 μm) solid-phase material manually packed into 6-ml polypropylene cartridges (Supelco, Toronto, Ont., Canada). The cartridges were conditioned by sequentially eluting with 6 ml *n*-hexane, 3 ml acetone, 6 ml methanol, and 2 ml dechlorinated water adjusted to pH 2.0 with 3.5 *M* H_2SO_4. The packing was not allowed to go dry until the end of the sample extraction step.

Prior to the extraction, each effluent sample was vacuum filtered through 0.45-μm glass-fiber filters, which had been pre-washed with hexane and dichloromethane in a Soxhlet apparatus. After filtration, the water samples were acidified to a pH of 2.0 with 3.5 *M* H_2SO_4, mixed well, and then immediately passed through the SPE cartridges at a rate of approximately 20 ml/min. The sample bottles were rinsed with 10 ml of pH 2.0 distilled water, and the rinses were allowed to flow through the cartridges. After passage of the samples, the cartridges were dried for 1 h under vacuum and then extracted by eluting with three successive 1-ml aliquots of methanol. Each aliquot of methanol was eluted through the column for a minimum of 10 min. The eluates were collected in a 10-ml collection tube, and were concentrated to 0.2 ml with a Universal Vacuum System UVS 400 (Savant Instruments, Inc.).

2.3. LC separation and ESI-MS–MS analysis

Analyses were carried out using a Waters 2690 HPLC equipped with a Genesis C_{18} column (150×

Table 1
Names, CAS registry numbers, chemical structures, and use and origin of acidic pharmaceuticals

Compound	Structure	CAS RN MW[a] Formula	Use/origin
Bezafibrate		41859-67-0 361.11 $C_{19}H_{20}ClNO_4$	Lipid regulator
Clofibric acid		882-09-7 214.04 $C_{10}H_{11}ClO_3$	Metabolite of lipid regulator, clofibrate, etofyllin clofibrate
Diclofenac		15307-86-5 295.02 $C_{14}H_{11}Cl_2NO_2$	Antiphlogistic
Fenoprofen		31879-05-7 242.09 $C_{15}H_{14}O_3$	Analgesic/antiphlogistic
Gemfibrozil		25812-30-0 250.16 $C_{15}H_{22}O_3$	Lipid regulator
Ibuprofen		15687-27-1 206.13 $C_{13}H_{18}O_2$	Analgesic/anti-inflammatory
Indomethacin		53-86-1 357.08 $C_{19}H_{16}ClNO_4$	Analgesic/anti-inflammatory
Ketoprofen		22071-15-4 254.09 $C_{16}H_{14}O_3$	Analgesic/anti-inflammatory
Mecoprop[b]		93-65-2 214.04 $C_{10}H_{11}ClO_3$	Herbicide
Naproxen		22204-53-1 230.09 $C_{14}H_{14}O_3$	Analgesic/anti-inflammatory

[a] Monoisotopic molecular mass.
[b] Internal standard.

2.1 mm, I.D., particle size 4 μm) purchased from Jones Chromatography, Hengoed, Mid-Glamorgan, UK, and a C_{18} guard cartridge purchased from Phenomenex, Torrance, CA, USA. The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (acetonitrile/methanol, 40:60) and B (20 m*M* aqueous ammonium acetate) at a flow-rate of 0.2 ml/min. The gradient was operated from 60 to 100% eluent A for 8 min, then held at 100% eluent A for 7 min. The washing solution for the autosampler was composed of 50% acetonitrile and 50% water. The solvents were degassed by an in-line degasser. A volume of 25 μl was injected and the LC effluent was directed to the ESI source without splitting.

All analyses were carried out on a Micromass Quattro LC triple quadrupole mass spectrometer equipped with a Z-spray electrospray interface (Manchester, UK) in negative-ion mode. Instrument control, data acquisition and evaluation were done with Masslynx NT software (v 3.4). The capillary and cone voltages were operated at 3.0 kV and 20 V, respectively. The temperatures of the electrospray source and nebulizing gas were 80 and 300 °C, respectively. Nitrogen was used as both nebulizing gas and desolvation gas at flow-rates of 70 and 600 l h^{-1}, respectively. Individual standard solutions were infused through a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow-rate of 10 μl/min into the mass analyzer. Following the selection of precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out using 2.0×10^{-4} mbar UHP argon (Praxair Products Inc., Peterborough, Ont., Canada) in the hexapole collision cell at collision energies in the range 20–70 eV. Product ion mass spectra were obtained at a series of collision energies to characterize each compound's fragmentation pattern and to select appropriate instrument parameters that produced a useful abundance of fragment ions for each compound. Optimal collision energy was determined and was set for each analyte.

The multiple reaction monitoring (MRM) transition channels, together with their collision energies and product ions are listed in Table 2. The mass spectrometer was operated in MRM mode with unit mass resolution on both the first and second analyzers. A dwell time of 200 ms per ion pair was used. The herbicide mecoprop was used an internal standard (1.0 μg/ml) because of its similar structural and chromatographic properties to the analytes, and it was added to each extract to monitor the LC–ESI-MS–MS analysis procedure. Procedural blanks were carried out through the whole analytical procedure to check for interference and contamination.

2.4. Validation of the analytical procedure

The extraction recoveries of the analytes from the matrices were estimated using STP effluent spiked with analytes at a concentration of 0.1 μg/l. The concentrations of the spiked samples were calculated from the calibration curve and compared to the theoretical values in order to calculate the extraction recoveries. Unspiked STP effluent was tested for the absence of interfering compounds and the specificity of the method.

The linearity of the method was evaluated for the analytes using STP effluent spiked with analytes in the concentration range of 0.01–10.0 μg/l. Experimental data fitted a linear mode, $y=a+bx$. The precision of the method was expressed as the relative standard deviation (RSD) of replicate measurements. In this work, precision was evaluated as both intra- and inter-day reproducibilities of the analytical method with two analyte concentrations, 0.2 and 1.0 μg/l. Several aliquots of each sample were tested the same day to determine the intra-day precision, and aliquots of the same sample were tested on different days to determine inter-day precision. The accuracy of the method was defined as the percentage of deviation from the known added amount analyte in the sample. The limit of detection (LOD) was defined as the minimum detectable amount of analyte with a signal-to-noise ratio of 3:1 in MRM mode.

3. Results and discussion

3.1. LC–ESI-MS–MS optimization

The $[M-H]^-$ ions of the analytes were observed in negative-ion mode as the base peaks in the mass spectra for all acidic drugs, and they were selected as precursor ions (Table 2). The extent of fragmentation of the product ions depends on the collision energy,

Table 2
Optimal LC–ESI-MS–MS (MRM) in negative-ion mode conditions for analysis of the acidic pharmaceuticals

Compound	Collision (eV)	MRM transition	Precursor and product ions	
Bezafibrate	65	360>274	360.1	$[M-H]^-$
			274.2	$[M-H-C_4H_6O_2]^-$
			154.2	$[M-H-C_{12}H_{14}O_3]^-$
			85.4	$[C_4H_5O_2]^-$
Clofibric acid	35	213>127	213.0	$[M-H]^-$
			127.2	$[C_6H_4ClO]^-$
			85.3	$[C_4H_5O_2]^-$
Diclofenac	50	294>250	294.0	$[M-H]^-$
			250.2	$[M-H-CO_2]^-$
Fenoprofen	35	241>197	241.1	$[M-H]^-$
			197.2	$[M-H-CO_2]^-$
			93.3	$[M-H-C_9H_8O_2]^-$
Gemfibrozil	45	249>121	249.2	$[M-H]^-$
			121.3	$[M-H-C_7H_{12}O_2]^-$
Ibuprofen	25	205>161	205.2	$[M-H]^-$
			161.3	$[M-H-CO_2]^-$
Indomethacin	25	356>312	356.0	$[M-H]^-$
			312.2	$[M-H-CO_2]^-$
			297.3	$[M-H-C_2H_3O_2]^-$
Ketoprofen	40	253>209	253.1	$[M-H]^-$
			209.3	$[M-H-CO_2]^-$
Mecoprop	65	213>141	213.0	$[M-H]^-$
			141.1	$[M-H-C_3H_4O_2]^-$
Naproxen	30	229>170	229.1	$[M-H]^-$
			185.2	$[M-H-CO_2]^-$
			170.2	$[M-H-C_2H_3O_2]^-$

and on the collision gas pressure in the hexapole collision cell between the first and second quadrupoles of the mass spectrometer. In practice, it is simpler to optimize the collision gas pressure and keep it constant during ESI-MS–MS experiments, and to control the CID by varying the collision energy via the applied voltage difference in the collision cell.

The product ion mass spectra for each compound were recorded in order to select the most abundant mass to charge ratios (m/z) for further study. The product ions generated at various collision energies are shown in Table 2. The product ions generated by expulsion of CO_2 were the only fragment ions formed during the CIDs for diclofenac, ibuprofen and ketoprofen. More structurally informative fragment ions could be observed in the product ion spectra of the other analytes. Table 2 also shows the selected MRM channels of the pharmaceuticals investigated, which were used for LC–ESI-MS–MS analysis of these compounds.

Initially, both acetonitrile and methanol were tested as organic mobile phases for the LC separation. The measurements were finally carried out with a mixture of acetonitrile and methanol (40:60) as organic mobile phase, which led to shorter retention times and better resolution of the analytes. Ammonium acetate was used as a mobile phase additive to improve ESI performance in negative ion mode. The mobile phase program was described earlier. Good separation quality and peak shapes were achieved for the compounds with a total run time of 18 min.

3.2. Linearity, recovery and sensitivity

Calibration curves were prepared for each compound from the spiked samples by plotting the average total ion peak area versus the analyte concentration. Table 3 displays the mean correlation coefficients (r^2) of the calibration curves, which are

Table 3
Mean percent recoveries (%), limits of detection (LODs) (ng/l) and linearity (r^2) of acidic pharmaceuticals determined by analysis of spiked STP effluent

Compound	STP effluent		
	LOD	Recovery	r^2
Bezafibrate	10	67.1	0.999
Clofibric acid	10	82.2	0.998
Diclofenac	10	62.8	0.997
Fenoprofen	10	91.5	0.993
Gemfibrozil	5	78.2	0.995
Ibuprofen	5	71.0	0.992
Indomethacin	10	58.5	0.993
Ketoprofen	20	83.9	0.996
Naproxen	10	68.4	0.994

higher than 0.992 in the STP effluent samples, indicating good linearity.

The recoveries of the analytes from the spiked effluent samples at a concentration of 0.1 μg/l ranged from 58.5 to 91.5% (Table 3). At this spiked concentration, the highest recovery was for fenoprofen at 91.5%, while the lowest recovery was for indomethacin at 58.5%.

The calculated LODs of the acidic drugs from the matrices are also shown in Table 3, and they ranged from 5 to 20 ng/l in spiked STP effluent. Ketoprofen showed relatively poor LOD, but is still comparable to those reported previously for GC–MS–MS analysis [10]. Gemfibrozil and ibuprofen gave very low LODs in this study.

3.3. Precision and accuracy

The ability of LC–MS–MS instrumentation to provide stable and reproducible ion currents over an extended period of time is of great importance. Precision was investigated by observing the short-term and long-term RSDs under identical conditions. The results for intra- and inter-day precision are presented in Table 4, and they were 6.9–11.4 and 7.6–11.7%, respectively, in the spiked STP effluent. The RSDs representing accuracy are also shown in Table 4, which were 4.9–11.5%.

3.4. Specificity

Spiked samples were studied for interference from compounds in the STP effluent. Fig. 1 illustrates a chromatogram for STP effluent spiked with analytes at a concentration of 0.05 μg/l. Two interfering

Table 4
Relative standard deviation (%) of replicate analysis to determine accuracy, and intra- and inter-day precision of the LC–MS–MS method for the spiked STP effluent, calculated at two concentrations (0.2 and 1.0 μg/l)

Compound	Concentration (μg/l)	Accuracy[a] (%)	Precision (%)	
			Intra-day	Inter-day
Bezafibrate	0.2	8.9	10.5	11.1
	1.0	7.3	8.1	10.2
Clofibric acid	0.2	9.2	9.7	8.9
	1.0	8.1	8.6	8.4
Diclofenac	0.2	7.8	9.7	10.1
	1.0	8.9	7.9	9.2
Fenoprofen	0.2	11.0	10.3	11.7
	1.0	10.2	8.7	9.2
Gemfibrozil	0.2	4.9	7.1	7.6
	1.0	6.2	6.9	8.1
Ibuprofen	0.2	9.7	9.6	10.3
	1.0	8.1	10.2	8.5
Indomethacin	0.2	10.5	9.5	10.9
	1.0	8.8	8.8	8.7
Ketoprofen	0.2	11.5	9.7	9.3
	1.0	9.2	8.1	9.0
Naproxen	0.2	8.7	11.4	10.2
	1.0	9.5	10.2	9.2

[a] Accuracy is defined as % deviation from ''added concentration''.

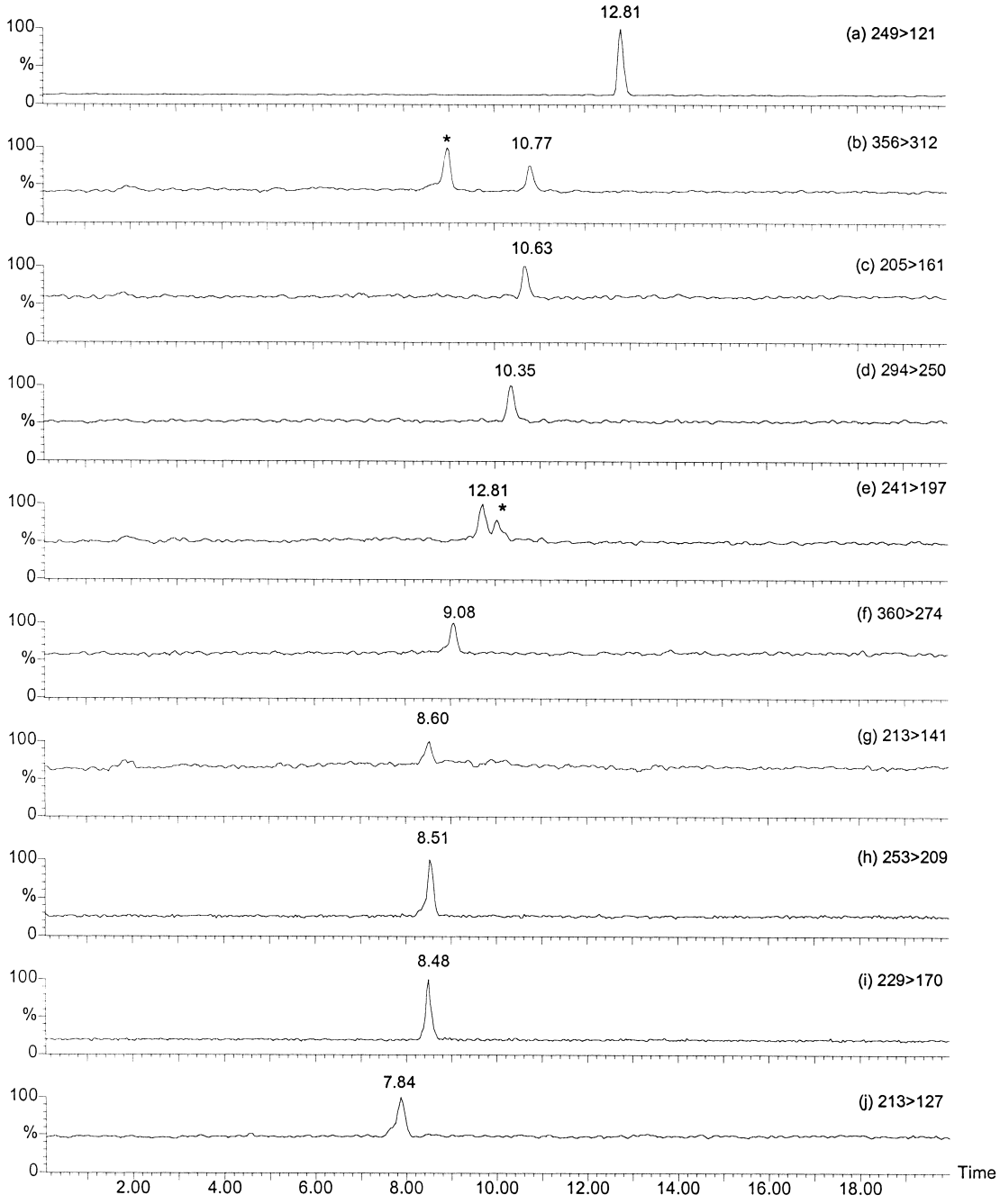


Fig. 1. Multiple reaction monitoring (MRM) chromatograms of spiked STP effluent (0.05 μg/l). Interfering peaks are marked by asterisks. (a) Gemfibrozil; (b) Indomethacin; (c) Ibuprofen; (d) Diclofenac; (e) Fenoprofen; (f) Bezafibrate; (g) Mecoprop; (h) Ketoprofen; (i) Naproxen; (j) Clofibric acid.

peaks (marked by asterisks) resulting from endogenous components in the STP effluent were shown in the MRM channels for fenoprofen and indomethacin. Coextractives in the STP effluent yield fragmentation patterns similar to fenoprofen and indomethacin. However, the separation efficiency provided by HPLC was sufficient to resolve these interfering compounds from the analytes. The importance of using chromatographic separation to improve assay selectivity was demonstrated in the analysis.

3.5. Analytical applications

Pharmaceuticals are subject to metabolism in organisms, such as hydroxylation, cleavage or glucuronidation. However, a significant amount of the original substance is excreted unmetabolized via urine or feces and enters STPs [11,18]. The presence of pharmaceutical residues in the aquatic environment clearly shows that elimination in municipal STPs is often incomplete [9,18]. The method developed in this study was applied to determine acidic pharmaceuticals in STP effluents in order to evaluate its suitability for analysis of environmental samples. In the three STP final effluent samples, the sample matrix did not significantly affect analyte ionization. Fig. 1 illustrates a spiked sample. Several acidic pharmaceuticals were detected in the STP effluent samples, and Fig. 2 illustrates the mean concentrations and relative standard deviations (triplicate). In general, the profiles of the drugs detected in STP-1 and STP-2 from Whitby, Ontario are very similar. However, the distribution of pharmaceuticals in STP-3 from Peterborough was different from those of STP-1 and -2. Indomethacin was detected and naproxen was present at very high concentrations in the effluent of STP-3. Clofibric acid and ketoprofen were not detected in effluent samples.

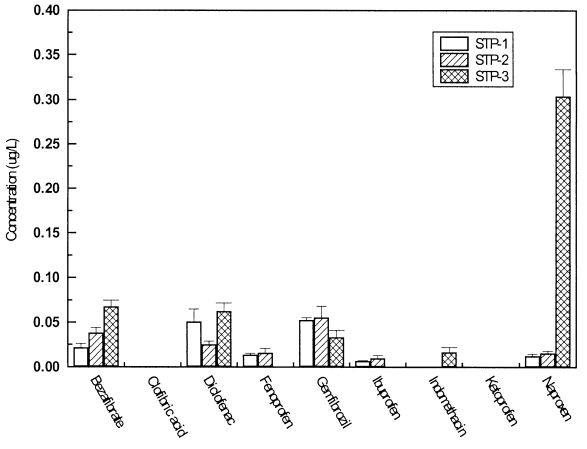


Fig. 2. Mean concentrations and RSDs ($n=3$) of acidic drugs in the final effluents from two STPs in Whitby (STP-1 and -2) and one STP in Peterborough (STP-3), Ont., Canada.

Conclusions

The LC–MS–MS method developed in this study for analysis of acidic pharmaceuticals gives good chromatographic resolution and ion intensity for these compounds. The method provides acceptable recoveries and LODs for acidic pharmaceuticals in STP effluent matrices. This method can be used to determine ng/l levels of acidic pharmaceuticals in aqueous samples without any derivatization steps.

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References

[1] H.-R. Buser, M.D. Müller, N. Theobald, Environ. Sci. Technol. 32 (1998) 188.
[2] H.-R. Buser, T. Poiger, M.D. Müller, Environ. Sci. Technol. 32 (1998) 3449.
[3] C. Hartig, T. Storm, M. Jekel, J. Chromatogr. A 854 (1999) 163.
[4] T. Poiger, H.-R. Buser, M.D. Müller, Environ. Toxicol. Chem. 20 (2001) 256.
[5] C.G. Daughton, T.A. Ternes, Environ. Health Perspect. 107 (1999) 907.
[6] D.L. Sedlak, J.L. Gray, K.E. Pinkston, Environ. Sci. Technol. 34 (2000) 509A.
[7] F. Stuer-Lauridsen, M. Brikved, L.P. Hansen, H.-C. Holten Lützhøft, B. Halling-Sørensen, Chemosphere 40 (2000) 783.

[8] L. Wollenberger, B. Halling-Sørensen, K.O. Kusk, Chemosphere 40 (2000) 723.
[9] T.A. Ternes, Water Res. 32 (1998) 3245.
[10] T.A. Ternes, M. Stumpf, B. Schuppert, K. Haberer, Vom Wasser 90 (1998) 295.
[11] C.D. Metcalfe, B.G. Koenig, T.A. Ternes, R. Hirsch, Environ. Toxicol. Chem. 2001, submitted.
[12] T.A. Ternes, R. Hirsch, J. Mueller, K. Haberer, Fresenius J. Anal. Chem. 362 (1998) 329.
[13] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwanz, K.-L. Kartz, J. Chromatogr. A 815 (1998) 213.
[14] F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn, J. Chromatogr. A 938 (2001) 199.
[15] M. Moeder, S. Schrader, M. Winkler, P. Popp, J. Chromatogr. A 873 (2000) 95.
[16] M. Farré, I. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, D. Barceló, J. Chromatogr. A 938 (2001) 187.
[17] W. Ahrer, E. Scherwenk, W. Buchberger, J. Chromatogr. A 910 (2001) 69.
[18] M. Stumpf, T.A. Ternes, R.-D. Wilken, S.V. Rodrigues, W. Baumann, Sci. Total Environ. 225 (1999) 135.