Application of gas chromatography-hybrid chemical ionization mass spectrometry to the analysis of diclofenac in wastewater samples

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Abstract

Hybrid chemical ionization (HCI), a new and useful alternative to conventional chemical ionization mass spectrometry, has been applied to the analysis of the pharmaceutical diclofenac in wastewater samples. This technique takes advantage of the high versatility of ion trap (IT) spectrometers combined with external ionization sources. In hybrid configuration, reagent ions are generated in the external source through electron ionisation (EI) of a reagent gas. These reagent ions are then drawn into the ion trap and only those selected are allowed to react with analytes eluting from the GC column. These ion-molecule reactions create analyte ions which are held in the ion trap. In this study ion-molecule reactions between C3F5+ cations, generated from perfluorotributylamine (FC43), and diclofenac molecules have been investigated. The observed reaction products were [M + C3F5–H2O]1+ adduct ions, which result from the initial electrophilic addition of C3F5+ cations to the diclofenac molecule followed by the rapid loss of H2O. Further fragmentation of these ions by MS/MS yielded enough daughter ions for a reliable identification of diclofenac in complex matrices. The GC–HCl–MS/MS method applied to wastewater samples provided highly enhanced selectivity and sensitivity, with a detection limit in real samples of 3.0 ng/L, for a solid-phase extraction (SPE) pre-concentration factor of 400. Other performance characteristics of the method, such as linearity and precision were also satisfactory. Finally, the method was successfully applied to the analysis of wastewater samples taken from the effluent of an urban sewage treatment plant (STP).

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1. Introduction

Chemical ionization mass spectrometry is a widely used analytical technique, which is recognized for the improved selectivity and sensitivity that can be achieved in the detection of several compounds [1,2]. In chemical ionization new ionized species are formed when gaseous analyte neutral molecules interact with reagent gas ions. These gas-phase ion-molecule reactions involve proton transfer, anion/proton abstraction, charge exchange and electrophilic addition that mainly form [M + H]1+ and [M–H]− quasimolecular ions of high abundance which enhances analyte detectability. Besides the advantages of this ionization technique a limitation in its application is the scarce number of reagent gases really available, even when the specificity of the method is highly dependent of the reactive gas used. Numerous studies have however been conducted to determine the reactivity of diverse ions in gas-phase [3–6] and, although these studies mainly have a theoretical interest, might be also useful from an analytical point of view. Thus, Mosi et al. [7] investigated ion/molecule reactions between fluorocarbon cations, generated from perfluorotributylamine (FC43), and polycyclic aromatic hydrocarbons (PAHs). Using this ion-molecule reaction, the differentiation of PAH structural isomers was possible [7]. Other applications focused to the differentiation of isomers have also been described in the literature [8,9].

Although Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR–MS) has been in many cases the analytical technique preferably used in the studies of the gas-phase ion chemistry [6,10] the advantages of ion trap (IT) mass spectrometers regarding sensitivity, versatility and low cost have led some authors to investigate the possible application of these instruments to this kind of studies, proving to be a valuable tool for this objective [7,11,12]. Despite the success of these investigations a commercial device including hardware and software specifically
addressed to the application of these specific ion/molecule reactions for analytical proposes had not been commercially available until recently. New ion trap instruments incorporate this capability as a new operation mode, which has been called hybrid chemical ionization (HCl), allowing an easy optimization and routine application of this new technique.

The HCl mode requires the use of an external ionization source in which the ionization of the reactive gas takes place by electron ionisation (EI). From the reagent ions generated, only those which are selected are stored in the ion trap. These trapped reagent ions are allowed to react with sample molecules, which enter the ion trap directly from the GC column through the transfer line, forming CI product ions. This approach has a number of potential advantages, including avoiding ion-molecule reactions with the neutral reagent and avoiding losses of negative ions that occur when they move from the external source to the trap. On the other hand, the adequate selection of a specific reagent gas ion allows improving the selectivity of the analytical method, especially in highly complex matrices. However, to our knowledge, analytical applications of this technique have not been published and therefore its performance characteristics have not been evaluated for routine application.

The aim of this work has been develop and validate an analytical method, based on the use of HCl to the analysis of diclofenac, a non-steroidal anti-inflammatory drug commonly used as an analgesic, antiarthritic and antirheumatic, which presence has been reported in natural water and in wastewater treatment plant effluents as a consequence of its incomplete elimination with conventional wastewater treatments.

2. Materials and methods

2.1. Chemicals

Diclofenac sodium salt was purchased from Sigma–Aldrich (Steinheim, Germany). Pesticide-grade ethyl acetate was from Panreac (Barcelona, Spain) and HPLC-grade water and methanol from Merck (Darmstadt, Germany). Perfluorotributylamine (PFTBA, commonly known as FC43 or heptacosa) was provided by Varian (Walnut Creek, CA, USA). Diclofenac stock standard solution was prepared in methanol from Merck (Darmstadt, Germany). Perfluorotributylamine (PFTBA, commonly known as FC43 or heptacosa) was provided by Varian (Walnut Creek, CA, USA). Diclofenac sodium salt was purchased from Sigma–Aldrich (Steinheim, Germany). Pesticide-grade ethyl acetate was from Panreac (Barcelona, Spain) and HPLC-grade water and methanol from Merck (Darmstadt, Germany). Perfluorotributylamine (PFTBA, commonly known as FC43 or heptacosa) was provided by Varian (Walnut Creek, CA, USA).

Diclofenac stock standard solution was prepared in methanol and stored at –20 °C. For GC–MS determinations, working standard solutions, at different concentrations, were prepared by appropriate dilution of the stock solution in both pure ethyl acetate and blank extracts of wastewater samples. In the last case, 200 µL aliquots of the wastewater extract were evaporated to dryness under a gentle stream of nitrogen and dissolved again, with sonication, in 200 µL of ethyl acetate containing the diclofenac standard.

2.2. Sample collection and preparation

Wastewater samples used in this study were collected from the final effluent of a municipal sewage treatment plant (STP). Samples were collected by using pre-rinsed amber glass bottles. After collection, samples were filtered through a 0.7 µm glass fibre filter (Teknokroma, Barcelona, Spain) prior to analysis, in order to remove particles that may interfere during the extraction procedure.

A solid-phase extraction (SPE) procedure was applied to the wastewater samples using commercial Oasis HLB (divinylbenzene/N-vinylpyrrolidone copolymer) cartridges (200 mg, 6 cm3) from Waters (Milford, MA, USA). An automated sample processor ASPEC XL fitted with an 817 switching valve and an external 306 LC pump from Gilson (Villiers-le-Bel, France) was used for this purpose. Conditioning step was performed with 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of LC-grade water, acidified at pH 3 with HCl, at a flow rate of 1 mL/min. Wastewater samples (400 mL effluent) were acidified at pH 3 with HCl and loaded at a flow rate of 10 mL/min followed by a washing step with 6 mL of water (pH 3). After that, the cartridges were dried by nitrogen stream during approximately 15 min and finally eluted with 2 mL × 4 mL of ethyl acetate at 1 mL/min. The extracts so obtained were evaporated by a gentle nitrogen stream until a final volume of 1 mL for direct analysis by GC–MS.

2.3. Chromatographic determinations

All experiments were performed on a Varian 4000 GC/MS/MS system equipped with external ionization source. Automatic injections (1 µL) were performed in a split/splitless injector at 250 °C, working in splitless mode with a splitless time of 1 min. Separations were performed in a crosslinked 5%-phenyl-95%-dimethylpolysiloxane Varian FactorFour (VF-5 ms, Varian, Middelburg, The Netherlands) capillary column (30 m, 0.25 mm, 0.25 µm), using the following oven program: 1 min at 70 °C, first ramp at 30 °C/min to 200 °C, second ramp at 2 °C/min to 220 °C (held for 2 min) third ramp at 10 °C/min to 300 °C (held for 2 min). The helium carrier gas flow was maintained at the constant value of 1 mL/min.

The GC–MS interface and the ion trap temperature were set at 250 and 200 °C, respectively. Data acquisition and processing, and instrumental control were performed by the Varian MS Workstation Version 6.42. Typical IT–MS operating conditions were optimised by the software at the following values: electron multiplier at 1125 V, trap offset at 7 V, lens 1 at 35 V, lens 3 at 23 V and gate lens at –108 V. The external ion source worked in CI mode (FC43 as reagent gas) at a temperature of 200 °C. Specific details on the use of the ion trap in HCl mode are described below (Section 3). The most important HCl parameters were set as it is shown in Fig. 1. MS/MS conditions for the collision-induced dissociation (CID) of the selected precursor ion were optimized using the automated method development (AMD) option. Optimized values were: precursor ion, m/z 410; isolation window, 4; isolation time, 10 ms; waveform type, non-resonant; storage level (m/z) 179.7; excitation amplitude, 119 V; excitation time, 15 ms.

2.4. Validation studies

Performance characteristics of the proposed method were established by using wastewater extracts taken from the STP effluent. Linearity was assessed with both pure solvent and
matrix-matched calibration curves, in order to evaluate matrix effects. Calibration solutions were prepared at six concentration levels, ranging from 0.10 to 4.0 μg/L. Each point was obtained as the average of two injections. Integrated peak area data of three selected quantification masses (m/z 242, 408 and 410) were used to construct the curves. The recovery studies (n = 4) were carried out by spiking sewage samples at the concentration level of 1 μg/L. Precision studies were performed by the repeated injection of a spiked extract during the same day (n = 10, repeatability) and in different days (n = 10, reproducibility). Since diclofenac was present in all the wastewater samples analysed, the limit of detection (LOD) was determined by extrapolation from the initial concentration present in the samples. This concentration was determined by the standard addition procedure and then the LOD calculated by extrapolating from the corresponding signal-to-noise ratio to a value of 3.

3. Results and discussion

3.1. Hybrid chemical ionization function

New GC–MS/MS systems incorporate software capabilities to allow the optimization and control of specific HCl parameters. Throughout this process the ion trap operates in a pulsed mode, following a three-stage sequence:

(a) Ionization. During this period the reagent gas is ionized in the external source and the selected reagent ions are stored in the ion trap. Ionization time is determined by a pre-scan function.
(b) Reaction. Reagent gas ions react with sample molecules into the trap to form sample adducts. Reaction time is also determined by a pre-scan function.
(c) Mass analysis. Reagent ions are ejected from the trap and afterwards the hybrid CI mass spectrum is acquired for the sample ions.

Fig. 1 shows a diagram representing the hybrid CI scan function.

3.2. Optimization of HCl parameters

Perfluorotri-n-butylamine is a reagent commonly used as a calibration compound in electron ionization mass spectrometry. The spectrum obtained under EI shows the characteristic fragment ions at m/z 69 [CF₃]+, 131 [C₃F₅]+, 264 [C₄F₁₀N]+, 414 [C₅F₁₅N]+ and 614 [C₁₂F₂₄N]+ used for routine calibration purposes. These ions are relatively stable but, it has been demonstrated that in the gas-phase, they may exhibit ion-molecule reactions to form corresponding mass adducts. An example of this reactivity has been referenced by He et al. [6], which describe
ion-molecule reactions of PFTBA with pyridine. Furthermore, the fragment ion at $m/z$ 69 [$\text{CF}_3]^+$ has been shown to undergo electrophilic addition with a variety of compound classes, such as halogenated aromatics, pyrrole, furan, thiophene, alkyl benzenes, aromatic carboxyls, hydroxy and alkoxy benzenes and oxygen and nitrogen bases [13]. Due to the proven reactivity of these ions, and the availability of PFTBA as an appropriate standard sample, it was selected as the reagent gas to be used in this study.

The liquid reagent is placed into a vial placed at the back of the instrument and connected to the CI inlet by means of a 50 mL/min restrictor. The pressure of the reagent gas in the trap is manually controlled by a valve. It was observed that analyte response was highly dependent on the amount of reagent ions present in the trap. Therefore, the intensity of the reagent ion was manually adjusted and visualized on the screen (see Fig. 1) to obtain a maximum value. In addition to general parameters, those specific to HCI were optimized. Fig. 1 shows the screen used for this purpose and the values applied for each parameter. The PFTBA fragment ion at $m/z$ 131 was selected as the reagent ion and all parameters were set to optimize isolation and further reaction of this cation in the trap. Isolation was controlled by the reagent low mass, reagent high mass and ejection amplitude parameters. Reagent low mass and reagent high mass were set to separate the reagent ion. The reagent low mass sets the RF storage level to exclude ions below the selected $m/z$ and must be set at least 10 u below the mass of the reagent ion of interest ($m/z$ 120 in our case). The reagent high mass isolation step occurs after the ionization time, when resonant waveforms are applied to the ion trap endcaps, in order to eliminate ions with $m/z$ above the selected reagent high mass ($m/z$ 140). The voltage of the waveforms is set by the ejection amplitude value, which was fixed at 40 V. The effect of these parameters during isolation adjustments can be visualized by turning on the ion trap manually and observing the reagent ion (Fig. 1). CI reaction and mass acquisition are controlled by the max reaction time, reaction storage level and CI background mass parameters. The reaction time is determined by a pre-scan function, however, the maximum time allowed for HCl reaction (max reaction time) was adjusted to 100 ms. Although the intensity of the product ions depends on the reaction time, tests performed at higher values of this parameter did not produce an increase in sensitivity, rather a decrease in the signal was observed. The reaction storage level is the RF storage level in the ion trap during the reaction period and must be set below the $m/z$ of the reagent ion. On the contrary, the CI background mass is set to eliminate the reactive ions before mass analysis and it is usually set at or above the reagent high mass.

3.3. HCI analysis of diclofenac

Once the HCI parameters were assigned, experiments were carried out injecting a diclofenac standard solution. The fragmentation pattern of diclofenac in EI mode shows characteristic isotopic clusters at $m/z$ 277/279/281, 242/244 and 214/216, which correspond to the series of ions [$M-\text{H}_2\text{O}]^+$, [$M-\text{H}_2\text{O}-\text{Cl}]^+$ and [$M-\text{H}_2\text{O}-\text{Cl}-\text{CO}]^+$. Molecular ion of diclofenac is not visible in the spectrum, since the loss of 18 amu, corresponding to the dehydration [$M-\text{H}_2\text{O}]^+$ and subsequent intramolecular cyclization to form the 1-(2,6-dichlorophenyl)indolin-2-one, is highly favoured [14] (see Fig. 2).

HCI analyses were performed by selecting the cation at $m/z$ 131 as reagent ion. Once in the trap, cation/molecule reactions yielded the formation of diclofenac adduct ions. This can be observed in the HCI full scan spectrum showed in Fig. 3A, in which a base peak at $m/z$ 408, corresponding to the [$M+\text{C}_3\text{F}_5-\text{H}_2\text{O}]^+$ ion, is observed. The absence of the [$M+\text{C}_3\text{F}_5]^+$ ion suggests that the electrophilic addition of the reactive ion to the aromatic ring or to the nitrogen atom of diclofenac molecule, is followed by a rapid dehydration, as occurs in EI. The resulting elimination product formed by HCI [$M+\text{C}_3\text{F}_5-\text{H}_2\text{O}]^+$, is much less energetic than the corresponding [$M-\text{H}_2\text{O}]^+$ ion formed in EI, and no further fragmentation is observed. Although the adduct formation is the preferential reaction between [$\text{C}_3\text{F}_5]^+$ and diclofenac, appearance of the [$M+1]^+$ ion was observed at higher analyte concentrations ($\geq 1$ mg/L). Formation of MH$^+$ ions has been previously referenced as a product of ion/molecule reactions when FC43 is used as reagent gas [7]. Since the reactive ion lacks any protons, additional residual proton sources in the ion source ($\text{H}_2\text{O}$) have been pointed out as responsible of MH$^+$ ions formation.

Despite the lower response obtained by HCl compared to EI, the total absence of noise in the selected ion chromatograms (SICs) obtained under HCI allowed us to obtain very good signal-to-noise ratios, assuring accurate quantification at very low concentration levels. However, correct identification of diclofenac in real wastewater samples presented some drawbacks: the scarce structural information obtained under HCI (only the isotopic cluster of the [$M+\text{C}_3\text{F}_5-\text{H}_2\text{O}]^+$ fragment at $m/z$ 408–412 was present in the spectrum) and the presence of spectral interferences consequence of co-elution of diclofenac.
with matrix components (ion at m/z 386). In order to guarantee the unequivocal confirmation of diclofenac in the samples, a tandem mass spectrometry based method was applied. MS/MS conditions for the collision-induced dissociation of the selected precursor ions were optimized using the automated method development option. The ion at m/z 408 was selected as precursor ion and the isolation window adjusted in order to isolate the isotopic cluster. This selection permitted detection of the isotopic cluster related to the product ions. Optimization was performed, at the non-resonant conditions, to give the highest yield of the daughter ions but avoiding complete disappearance of the parent ions to get more confidence in the identification. Diclofenac mass spectrum obtained under HCl–MS/MS conditions is showed in Fig. 3B. Characteristic diclofenac fragment ions at m/z 277 and 242 were present, providing a reliable confirmation of diclofenac in the samples.
3.4. Validation and application to real samples

All the quantitative analyses were performed using mass fragments at \( m/z \) 242, 408 and 410 as quantification ions. Selection of three masses allows us to increase peak response. Although selection of the precursor ions do not represent a good option for quantification purposes regarding selectivity, the possibility of matrix contribution to these ions under HCI was hardly probable because of the high \( m/z \) of the adduct ions and the selectivity of the HCI technique. For identification purposes three qualifier ions at \( m/z \) 242, 408 and 277 were selected. Presence of the characteristic isotopic clusters corresponding to the chlorine atoms presents in the diclofenac molecule were also considered for a better assessment of its identity in the samples.

Linearity was studied in the range from 0.10 to 4.0 ppb, which includes the concentration levels usually present in real samples [15]. Pure solvent and matrix-matched standard calibration curves were obtained. Better linear correlation was observed with matrix-matched standards in the studied range \( (R^2 = 0.995) \). This is consequence of matrix effects in the chromatographic system [16], which induces a signal enhancement effect in presence of matrix components, especially patent at lower concentration levels. The calibration curve constructed using standard solutions prepared in ethyl acetate better fit a polynomial trend, where linear segments could be distinguish depending on the concentration range considered [16]. Recovery studies yielded 93 \( \pm \) 10% and the calculated limit of detection of diclofenac in wastewater samples, was 3.0 ng/L. The low LOD achieved in the developed method is consequence of the high efficiency of HCI–MS/MS methodology in suppressing matrix background.

Intra-day and inter-day precision was evaluated by the repeated analysis (\( n = 10 \)) of a wastewater extract spiked at 0.20 ppb concentration level. Precision in peak area of the quantification ions and in relative abundance for the qualifier ions was evaluated in order to assure the correct identification and quantification of diclofenac. Dispersion within the data was expressed as relative standard deviation (RSD). Very good precision was observed in the relative abundance of the qualifier ions, with RSDs lower than 18% in all cases. Repeatability in peak area was also very good with RSD of 2%. However, reproducibility study yielded a RSD of 23%. This higher variation in inter-day precision can be consequence of the variability observed in the reagent gas ion intensity over the time, which makes necessary a daily calibration to assure a more precise evaluation of diclofenac in the samples.

4. Conclusions

With this application, HCI has proved to be a simple and efficient alternative in gas chromatography–ion trap–mass spectrometric analysis. Formation of adduct ions of high \( m/z \) as a product of highly specific ion-molecule reactions, allows to increase the selectivity and sensitivity by improving the differentiation of the analyte from interfering and co-eluting compounds and suppressing matrix background. Further MS/MS fragmentation of the adduct ions provides characteristic product ion spectra, enough for a suitable analyte confirmation. HCI–MS/MS method also provides good performance characteristics regarding linearity and precision, especially in intra-day assays with RSD of 2%, and a limit of detection of 3.0 ng/L. Worse results were however obtained for the reproducibility making necessary a daily calibration.

This novel instrumental configuration opens new possibilities to the study of gas-phase ion-molecule reactions and can be a useful tool for the trace analysis of selected compounds in complex matrices.

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