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Classification of the ibuprofen active pharmaceutical ingredients by chemical patterns combining HPLC, ^{1}H-NMR spectroscopy and chemometrics: traceability of legal medicines

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Abstract

Introduction. Ibuprofen is one of the widespread used non-steroidal anti-inflammatory drugs. Ibuprofen active ingredient is manufactured in many sites located all around the world. The aim of this paper was to classify the geographical source of ibuprofen active pharmaceutical ingredients (APIs) from the legal market, based on chemical characteristics and its impurity pattern and to define a geographical fingerprint.

Methods. To classify ibuprofen in different geographical groups, the chemometrics by principal component analysis (PCA) and Cluster analysis was applied to HPLC, ^{1}H-NMR data of twenty-four samples of APIs from approved manufacturers located in different European and Asian countries.

Results. The PCA showed clearly two different geographical groups, based on particular patterns of European or Indian samples; the cluster analysis showed the similarity of group.

Conclusion. The chemometric analysis is an important tool for tracking the geographical origin of APIs. This could be useful to supplement the quality control ensuring safety of the medicinal products in legal market and dealing with the evolving changes of the illegal market.

Key words

- active pharmaceutical ingredient (API)
- ibuprofen
- HPLC
- ^{1}H-NMR
- chemometrics

INTRODUCTION

Traceability of medicinal products is meant as the full knowledge of the medicine route from manufacturing of the active pharmaceutical ingredients (APIs) and excipients to commercial distribution.

APIs from the legal chain have similar high-quality standards as they are manufactured through validated processes in authorized facilities according to the stringent good manufacturing procedures (GMP) rules and are released in line with regulatory approved specifications [1, 2]. Accordingly, it is more challenging to discriminate the geographical origin of legal active ingredients which are a quite homogeneous "clean" whole. Even though medicinal products containing regular APIs should not represent a critical safety issue, awareness of the sources helps in the risk-based evaluation for regulatory inspections of manufacturing facilities and in promoting fast effective action in case of unforeseen adverse events. Examples of undue events in the legal chain of APIs are the worldwide case of heparin adulterated by oversulfated chondroitin sulfate (OSCS) [3-5]; Valsartan containing impurities as N-nitrosodimethylamines (NDMA) and N-nitrosodiethylamines (NDEA) and, lately, Ranitidin and Metformin also contaminated by NDMA [6-9].

It is acknowledged that the origin of active substances can be traced by comprehensive analysis of their physical and chemical properties and their impurity pattern.

The possible analytical approaches for impurity and chemical profiling and characterization of APIs are based on chromatographic and spectroscopic methods (NMR, NIR, IR), or on the isotopic nucleus ratio by IRMS (isotope ratio mass spectrometry) [10-15]. All these approaches proved effective in locating the origin of APIs, even though APIs are usually manufactured from reagents and starting materials that are sourced

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from different locations. In any case, advanced analytical techniques produce a huge amount of information that are difficult to evaluate for relevance if considered separately without a chemometric approach. Chemometrics consists in the application of multivariate analysis to handle chemical or process information as a whole, thus highlighting concealed patterns and relationships in the collected data [16-19].

The output of chemometrics can be used as a predictive tool of the properties of the investigated samples or for descriptive purposes to evidence hidden connections and similarities in a group of samples. Results are applicable to research, quality control, manufacturing in the pharmaceutical industry and to fighting falsification. Since 2016 the European Pharmacopeia (Ph. Eur. 9.5 Ed.) has dedicated a general text to the chemometric methods applied to analytical data outlining the importance of chemometrics in the processing of data sets to obtain maximal chemical information on the investigated samples (Ph. Eur. 5.21 Chemometric methods applied to analytical data).

Ibuprofen is the most widespread non-steroidal anti-inflammatory drug (NSAID). It had a calculated turnover of 169.7 million (euros) with reference to the Italian market for the year 2018 and it represented the second active ingredient for the expense of self-medication in Italy among self-medication drugs [20]. Ibuprofen, as an active substance, is sourced from suppliers located all over the world. On the illegal side, it is well known that ibuprofen popularity triggers counterfeiting which implies unknown location of the manufacturing site. Therefore, traceability of ibuprofen manufacturing location increases knowledge of the quality and the safety of this medicinal substance with a high impact on public health in the general population.

The analysed ibuprofen samples were all compliant to the Ph. Eur. Monograph ibuprofen and were within the validity period. Chemometrics, by principal component analysis (PCA) method with multivariate data compression and cluster analysis, were applied to the HPLC and ^{1}H-NMR data sets. The outcomes demonstrated that it was possible to classify such a homogeneous group of samples according to their geographical source based on chromatographic and spectroscopy profiles.

This study reports the results of a chemometric analysis on twenty-four ibuprofen APIs from legally approved manufacturers located in different countries. The evaluation was carried on with the purpose of defining the specific chemical characteristics of ibuprofen manufacturing among several countries and tracing the APIs origin.

MATERIALS AND METHODS

Collection of samples

Twenty-four different batches of ibuprofen active substance were collected during the Italian post-marketing surveillance program. The APIs were produced by seven different manufactures, placed in different European and Asian countries. All the samples were compliant to the specific monograph of the European Pharmacopoeia and were within the validity period.

Analytical methods

Two analytical methods were performed for the analysis of ibuprofen samples: HPLC and ^{1}H-NMR. In this phase the variability of the methods was not explored.

HPLC

A Waters Alliance HPLC 2695 (Waters Corporation, Millford, MA, USA) separation module equipped with an UV-DAD Waters 2996 was used. The analytical procedure was based on the method reported in Ph. Eur. 9.5 monograph of ibuprofen (0721).

The column used was a Symmetry C18-endcapped (150×4.6 mm - 5 µm particle size), whereas, the gradient elution is showed in *Table 1*: the Phase A was composed by water-acetonitrile-phosphoric acid (600:400:0.5, v/v/v), and the Phase B by acetonitrile.

The other parameters settings were: 2 ml/min for flow rate, 30 °C for column temperature, 20 µl for Injection volume, 20 mg/ml in mobile phase A for sample concentration, and 214 nm for detection (Wavelength prescribed in the Ph. Eur. Monograph).

It was investigated if additional peaks were present at different wavelengths than those ones indicated by the Ph. Eur. Monograph. The chromatograms were evaluated at wavelengths between 210 and 400 nm, but no further peaks were detected and the intensity of known secondary peaks was not higher than that measured at 214 nm. Therefore, the considered wavelength was confirmed at 214 nm. The run time was set at 70 minutes.

^{1}H-NMR

A Bruker Avance NMR spectrometer (Bruker BioSpin Gmbh, Billerica, Massachusetts, USA) operating at a frequency of 400 MHz for protons (9.4 Tesla), equipped with a 5 mm BBI Z-GRD- ^{1}H-^{13}C probe head was used for recording the experiments.

For each experiment, 10 mg of ibuprofen were dissolved in 0.7 ml of DMSO-d6 99.80% and TMS and the solution was stirred for two minutes to complete dissolution. ^{1}H-NMR 1D experiments were run at temperature of 298.0 K, 32 scans were acquired with a delay time of 20 s, pulse was set at 90°, spectral window was 20 ppm.

Chemometric analysis

Dataset description and pre-processing

HPLC – For the qualitative and quantitative analysis, the chromatograms obtained at wavelength of 214 nm were considered and processed using Empower software version 3 (Waters Corporation, Millford, MA, USA).

Table 1
Gradient elution of HPLC

Time (minutes)	Phase A (per cent V/V)	Phase B (per cent V/V)
0-25	100	0
25-55	100-15	0-85
55-70	15	85

V/V: volume/volume; HPLC: high performance liquid chromatography.

Only peaks not detected in the blank run were considered in the analysis, and the disregard limit was set to 90 ppm based on S/N ratio >10 for each sample. Quantity (area under the peak in %) of each peak, identified by the retention times (RTs), was obtained by normalising relevant area to the area of ibuprofen peak (RT of 13.96 min). The RTs of ibuprofen and Ph. Eur. impurities peaks were well-known; further/other peaks were taken into account only if they fulfilled the aforementioned requirements. The area % of each peak was evaluated by Empower software.

NMR - ^{1}H-NMR data were processed by TopSpin 3.4 software (Bruker BioSpin Gmbh, Billerica, Massachusetts, USA), applying 0.3 Hz line broadening and calibration to the TMS signal at 0 ppm. Spectra were overlapped and only signals with S/N ratio > 3 not belonging to ibuprofen structure were considered for qualitative data evaluation.

Two datasets were analysed: quantitative measurements represented by the area percent of the HPLC peaks, and qualitative measurements reported as presence or absence of signals at specific chemical shifts for ^{1}H-NMR. The presence of signals was indicated by number 1, and the absence by number 0. The samples were divided *a priori* in two classes by geographical area of manufacturing, according to the information reported in the certificates of analysis of the samples. The classification was class 1 for European countries and class 2 for Asian countries. Six signals for HPLC chromatograms and 16 signals of NMR spectra were selected. In total, twenty-two measurements were obtained in the overall dataset (*Table 2*).

Table 2
Characteristics of measurements analysed for ibuprofen samples

Analytical methods	Signals for datasets	Measurements
HPLC	RT 3.51 RT 5.2 RT 8.7 RT 13.2 RT 48.13 RT 66.41	Area of signal (%)
^{1}H-NMR	7.93 ppm 7.91 ppm 7.44 ppm 7.42 ppm 7.13 ppm 2.19 ppm 1.90 ppm 1.55 ppm 1.53 ppm 1.45 ppm 1.21 ppm 1.14 ppm 1.11 ppm 1.09 ppm 0.97 ppm 0.76 ppm	Presence/Absence of signal

HPLC: high performance liquid chromatography; ^{1}H-NMR: proton-nuclear magnetic resonance;
RT: retention time; ppm, parts per million; area of signal, area under chromatographic peak (%).

At first, the data of each analytical method were evaluated separately; subsequently, the effect of overall data (HPLC plus ^{1}H-NMR) was estimated. HPLC data were pre-treated with scaling method to analyse the HPLC and NMR measurements in a unified database.

Chemometric methods

Two unsupervised methods were applied: principal component analysis (PCA) and cluster analysis. The PCA reduced the number of measurements in the principal components (PCs), which represented highest variability of datasets and classified the ibuprofen APIs between the two classes of different geographical manufacturing area. The cluster analysis was carried out to evaluate similarities among samples by Euclidian distance to indicate the presence or absence of natural groupings between analysed samples.

Software

Data pre-treatment, PCA and cluster analysis were performed using Matlab R2018b software (The Mathworks, Natick, MA, USA). The algorithms for PCA and cluster analysis were part of the PCA_toolbox for Matlab-version 1.5 of Milano Chemometrics and QSAR Research Group [21].

RESULTS

The manufactures were localized for 54.2% in Europe (Italy, UK and Germany), 45.8% in Asia (India and China). They were grouped in two classes: Europe (class 1) and Asia (class 2).

PCA and HPLC

About 87.98% of variance were explained by two principal components (PC1 and PC2). The PC1 represented the highest variation of the data (70.31%) and the PC2 represented the 17.67% of variation of data. The PCA scores plot showed a separation between class 1 and class 2. Class 2 was characterised by negative PC1 and negative PC2. The PCA loadings showed that the associated measurements with class 2 (Asian samples) were RT 3.51 minutes and RT 13.2 minutes (*Figure 1S, available online as Supplementary Material*). The peaks eluted at these RTs could be typical of Asian ibuprofen manufacturing.

PCA and ^{1}H-NMR

Two principal components represented the 84.88% of variance for ^{1}H-NMR dataset: PC1 represented 63.32% and PC2 21.56% of variation of the data. The PCA scores plot demonstrated; separation between class1 (European samples) and class 2 (Asian samples). The class 2 was characterised by positive PC2. The chemical shifts associated with class 2 were: 1.09 ppm, 1.11 ppm, 7.42 ppm, 7.44 ppm, 7.91 ppm and 7.93 ppm (*Figure 2S, available online as Supplementary Material).* The signals in the spectra at these chemical shifts could be characteristic Asian ibuprofen manufacturing.

PCA & HPLC and ^{1}H-NMR

The overall dataset was composed by 22 measurements. Using the PCA, with two PCs, the variance was

82.51% (57.29% and 25.22%, respectively for PC1 and PC2).

The PCA scores plot confirmed the separation between class 1 and class 2 of ibuprofen samples. Class 2 was represented by positive PC2. Only score S13, corresponding to the only Chinese sample, was classified differently in respect to the Asian class with a negative PC2. The loadings plot showed that the Asian classification was determined by RT 3.51 minutes and by signals at chemical shifts 1.09 ppm, 1.11 ppm, 1.21 ppm, 7.42 ppm, 7.44 ppm, 7.91 ppm and 7.93 ppm which had a positive PC2 (*Figure 1*).

Cluster analysis

Four groups were defined on the basis of chromatography and spectroscopy similarities of samples, and of their distance (*Figure 2*). The first homogenous group of samples was represented by cluster A, which showed to be less related to the other clusters. Cluster A was composed of European samples. These results highlighted its difference from the other groups, in particular from cluster D, composed of Indian samples. Closer related cluster B, represented by sample S8 only, shared similar characteristics with cluster A. Cluster C showed one Asian sample (S13 from China) together with one European sample (S24 from UK). The presence of Asian and European samples within the same group C suggests that some basic characteristics of ibuprofen manufacturing (i.e., starting material, intermediate of synthesis, other materials) could be similar between the two geographical areas. Establishing the similarity of these samples was not possible due to the

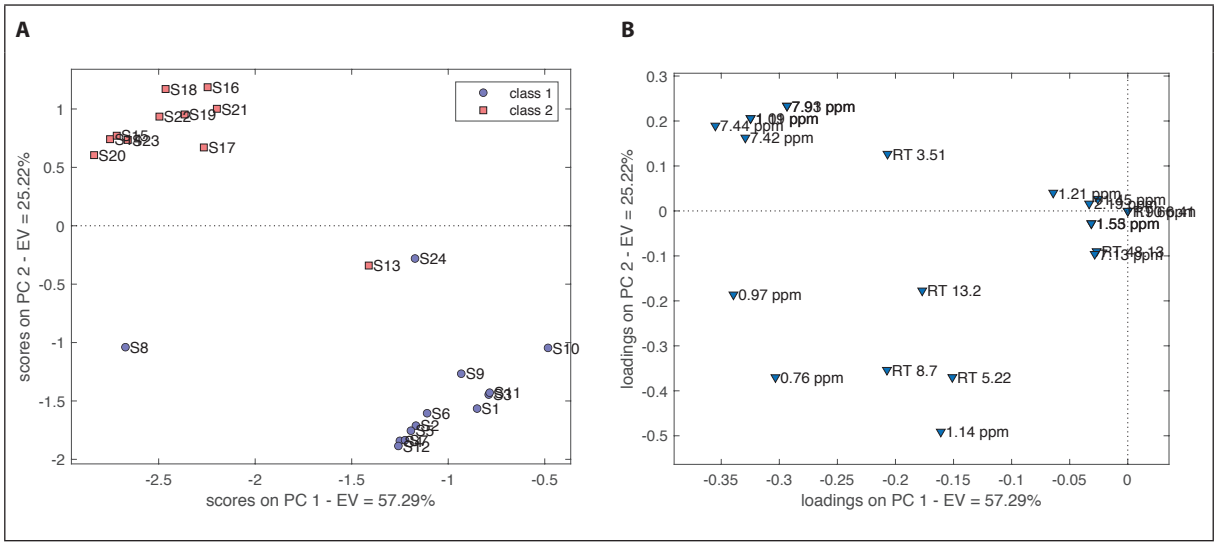

Figure 1
PCA scores plot (**A**) and loadings plot (**B**) of the overall dataset of the 22 combined HPLC and ^{1}H-NMR measurements.

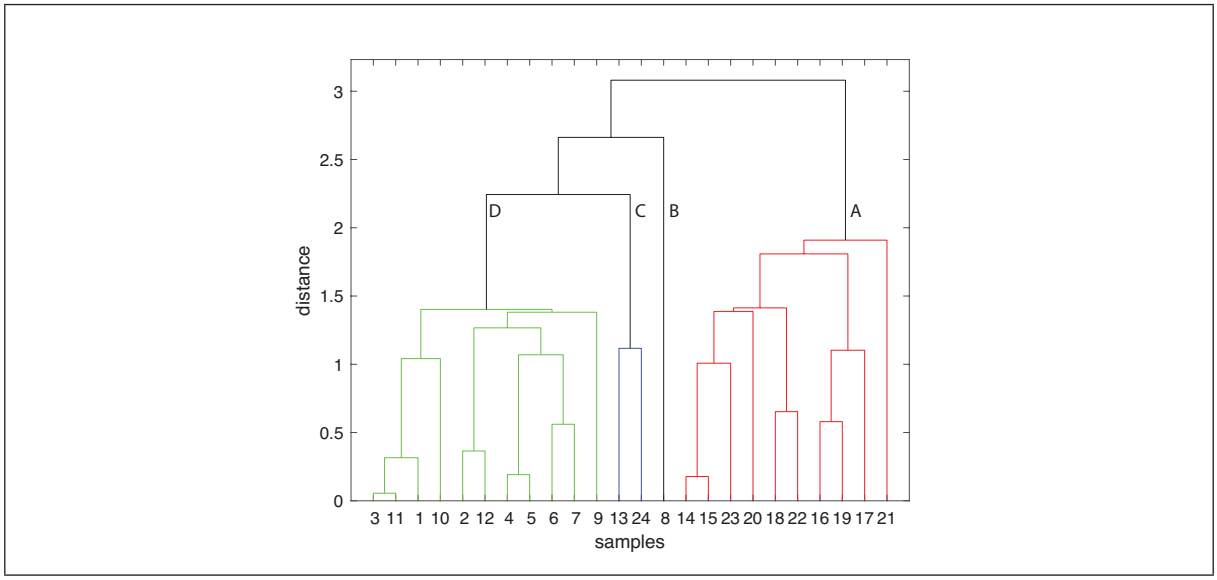

Figure 2
Dendrogram of cluster analysis for European and Asian samples.

low availability of samples for each country. Cluster D showed to be a homogenous group of samples, and it was represented by the Indian samples. These findings suggest that some characteristics of manufacturing of S13 (the only Chinese sample in Cluster C) could be different from the other Asian samples. For sample S24, the only ibuprofen sample manufactured in UK, the characteristics of manufacturing could be different from the other European countries.

DISCUSSION

This study demonstrated that marketed medicines containing active ingredient, such as ibuprofen, from different geographical origins, can be characterized by the active substance chemical and impurity profile. Indeed, the outcomes of this study demonstrated that by HPLC, ^{1}H-NMR and Chemometric tools, it is possible to classify APIs sharing the same chemical structure but being manufactured in different geographical areas. In fact, the first multivariate analysis, highlighted differences in legal ibuprofen APIs depending on manufacture geographical area. The PCA results, in fact, demonstrated that the European and Indian products were classified in two well distinct groups according to their different chromatography and ^{1}H-NMR spectroscopy fingerprints. The classification of samples improved when both HPLC and ^{1}H-NMR data were combined in the PCA analysis. Cluster analysis confirmed the classification in four clusters corresponding to the manufacturing region of ibuprofen. Hence, on the basis of the distance of the two relative clusters and of the chromatographic and spectroscopic profiles, the 24 samples were demonstrated to be not identical and that they could be classified in different homogenous groups.

Notwithstanding, the limited number of API samples, categorization between India and Europe was clearly noticeable.

Although the true root cause of the classification is unknown, some assumptions can be done. One hypothesis is that the purchase of the starting materials used for the API synthesis from manufacturers located in the same region of the world, would originate "similar" impurities pattern in the final API [22, 23]. Another possibility could be a different approach to GMP rules in the regulated countries. The possibility that the different patterns may be linked to different API manufacturing processes seems less convincing as the manufacturing processes to obtain ibuprofen are well known and established worldwide. However, this possibility cannot be completely ruled out as the choice of a manufacturing process can be based on the availability and cost of the starting materials which once again, may be linked to the region of origin.

CONCLUSION

The results of the present study confirmed chemometrics as a powerful predictive tool and stimulated further developments such as improving the geographical localization of the APIs source and to provide APIs fingerprints based on HPLC and ^{1}H-NMR. The chemometric analysis can be applied to different analytical method and can be useful to classify finished medicines or excipients. The new analytical methods provide huge amount of data which can be evaluated with chemometrics to better understand the characteristics of specific class of active substance.

The knowledge of the characteristics of the APIs manufactured in several areas of the world is also an important factor to oppose the illegal commerce of falsified medicines [16, 17]. Tracking geographical origin of APIs constitutes a major challenge to the OMCls either to assess the quality of legal medicines in post-marketing surveillance studies or to deal with the constantly evolving changes of the illegal market of falsified medicines. Furthermore, the knowledge of API fingerprint could be a very useful tool for Inspectorates to drive GMP inspections in particular geographical area.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

MR: conceptualization, methodology, data curation, software, roles/writing original draft. AB: conceptualization, methodology, formal analysis, roles/writing original draft. MB: formal analysis, roles/writing original draft. AM: resources. GG: conceptualization, methodology, formal analysis, roles/writing original draft.

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