Simultaneous Determination of Benzydamine Hydrochloride and Five Impurities in an Oral Collutory as a Pharmaceutical Formulation by High-Performance Liquid Chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of benzydamine hydrochloride and its impurities 3-dimethylaminopropyl 2-benzylaminobenzoate, 3-dimethylaminopropyl-2-aminobenzoate,1-benzyl-1H-indazol-3-ol, 1-benzyl-2-(3-dimethylaminopropyl)-1,2-dihydro-3H-indazol-3-one, and 1-benzyl-3-(3-(3-dimethylaminopropyl)-3-methylamino)propoxy-^{1}H-indazole in a collutory formulation is developed. The separation is carried out on a Gemini C_{18} (250 × 4.6 mm, 5 µm) column using acetonitrile–methanol–ammonium carbonate buffer (10 mM; pH 10.5) (37.5:37.5:25, v/v/v) as mobile phase at a flow rate of 1.0 mL/min, column temperature 30°C, and UV detection at 218 nm. Famotidine is used as an internal standard. The total run-time is less than 15 min. The analytical curves present coefficients of correlation greater than 0.99, and detection limits are calculated for all analytes. Excellent accuracy and precision are obtained for benzydamine hydrochloride. Recoveries vary from 98.25 to 102.8%, and intra- and inter-day precisions, calculated as the percent relative standard deviation, are lower than 2.2%. Specificity and robustness for benzydamine hydrochloride are also determined. The method allows the quantitative determination of benzydamine hydrochloride and its impurities, and it is suitable for routine analysis in quality control laboratories.

Introduction

Benzydamine hydrochloride (BZH) is a non-steroidal anti-inflammatory drug (NSAID) widely used for topical application. Its anti-inflammatory and analgesic properties along with possible side effects and contraindication are similar to those of drugs belonging to the same therapeutic class. However, topical use offers indisputable advantages in terms of safety. Benzydamine accumulates in the inflamed tissue and stabilizes the small vessels, which become more resistant to the harmful action of the substances formed during the inflammatory processes. With its membrane-stabilizing effect, benzydamine prevents white blood cells from releasing the enzymes, which would promote the inflammatory process. This drug exerts an anti-aggregating action on platelets and on the red blood cells. Benzydamine also exerts a relaxing action on the striated muscle and an antispasmodic action on the smooth muscles and has, therefore, a pain-killing effect. At higher concentrations, benzydamine inhibits the synthesis of specific substances called prostaglandins, which are actively involved in the inflammatory reaction determining the relative extent and duration and leading to the onset of the painful stimuli, which are typical of inflammation (1,2).

Benzydamine chemical name is N,N-Dimethyl-3-((1-(phenylmethyl)-1H-indazol-3-yl)oxy)-1-propanamine hydrochloride. The drug related impurities are 3-dimethylaminopropyl 2-benzylaminobenzoate (DAP), 3-dimethylaminopropyl-2-aminobenzoate (DABA),1-benzyl-1H-indazol-3-ol (BI), 1-benzyl-2-(3-dimethylaminopropyl)-1,2-dihydro-^{3}H-indazol-3-one (BDA), and 1-benzyl-3-(3-(3-dimethylaminopropyl)-3-methylamino)propoxy-1H-indazole (BDAP). The structures of benzydamine hydrochloride and its impurities are shown in Figure 1. There are several examples of published methods for the determination of benzydamine hydrochloride in biological fluids and in topical pharmaceutical preparations, which involve the use of (^{14}C)-benzydamine and liquid scintillation counting (3) or high-performance liquid chromatography (HPLC) with either UV (4,5) or fluorescence (6) or chemiluminescence (7) detection. An ion-selective piezoelectric sensor was used for determination of benzydamine in serum and urine (8). Recently a spectrophotometric method for its determination in bulk and pharmaceutical formulations was described (9). Studies of the photodegradation of benzydamine in pharmaceutical formulations by HPLC with diode array detection (10) and by an amperometric biosensor were reported (11). HPLC was also used for the determination of the impurity BI in benzydamine (12).

According to the FDA and ICH regulations, for assuring

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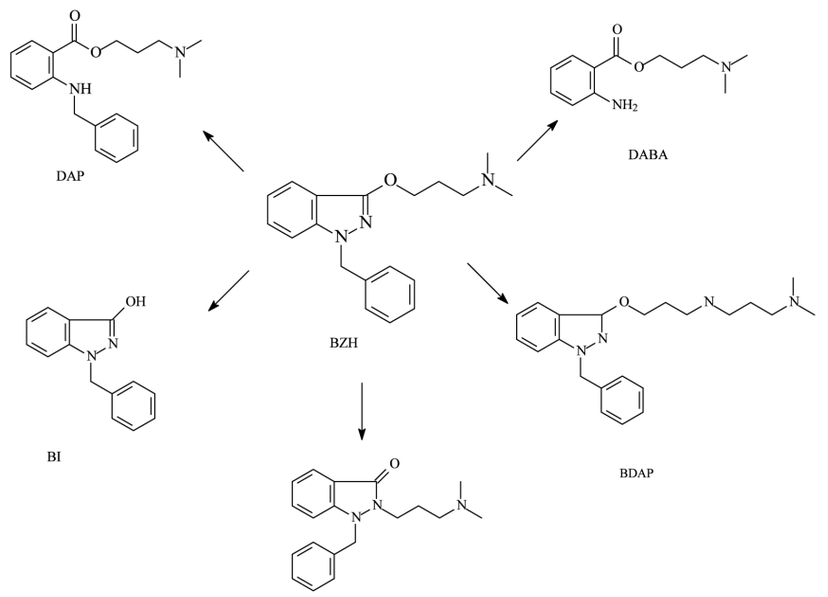

Figure 1. The structures of BDA, BI, DABA, benzydamine hydrochloride, BDAP, and DAP.

high quality of the commercial products, the determination of impurities in the drug substances and in the dosage forms is a critical part of the drug investigation process (13). In fact the safety of pharmaceuticals is dependent not only on the intrinsic toxicological properties of the active ingredient and excipients in the drug product, but partially upon the impurities that it may contain too (14,15). For example, to the benzydamine impurity BDA has been ascribed the benzydamine phototoxic effect towards human erythrocytes (16,17). No published study is available on the separation and simultaneous determination of benzydamine and its impurities in a collutory by HPLC or any other analytical technique. Consequently, the aim of this study was to develop and validate an isocratic chromatographic method, with UV detection, for the simultaneous evaluation of benzydamine and five of its impurities in a oral collutory.

Experimental

Instrumentation

The HPLC method was performed on a chromatographic system, consisting of a solvent delivery pump system (Waters, Milford, MA) model 600, a Rheodyne model 7125i injector (Rheodyne, Cotati, CA) fitted with 20-µL loop, an on-line degasing system (model DGU-14A Shimadzu Corporation, Kyoto, Japan) and an UV-Vis photodiode array detector model 2296 (Waters). The output signal was monitored and integrated using Empower software (Waters). A Gemini reversed-phase C_{18} column (250 mm × 4.6 mm i.d., 5 µm), protected by a disposable Security Guard Gemini C_{18} (3.0 mm × 4.0 mm i.d.) (Phenomenex, Torrance, CA), was used to produce the separation. A column thermostat oven module Igloo-Cil (Cil Cluzeau Info Labo, France) was used.

Chemicals

Acetonitrile and methanol (HPLC grade) were supplied by Fluka Chemika-BioChemika (Buchs, Switzerland). Ammonium carbonate, sodium hydroxide, and ammonia (HPLC grade) were obtained from Carlo Erba Reagenti (Milan, Italy). BI was purchased from Acros Organics (Carlo Erba, Milan, Italy). DAP, DABA, BDA, and BDAP were donated by Laboratory of Analytical Chemistry of the University of L'Aquila (L'Aquila, Italy). The famotidine, used in this study as an internal standard, uracil, and benzydamine hydrochloride were purchased from Sigma Aldrich (Milan, Italy). Water (HPLC grade) was obtained by passage through the ELIX 3 and Milli-Q Academic water purification systems (Millipore, Bedford, MA) and was used in the preparation of mobile phase buffer and reagent solutions.

Chromatographic conditions

The mobile phase was a mixture of acetonitrile, methanol, and ammonium carbonate buffer (10 mM; pH 10.5) (37.5:37.5:25, v/v/v) delivered at a flow rate of 1.0 mL/min at 30°C. The solvent was filtered before use through a 0.45-µm, WTP 0.5-µm membrane (Whatmann, Maidstone, U.K.), while ammonium carbonate solution was filtered through a WCN 0.45-µm membrane and degassed. The chromatograms were recorded at 218 nm.

Sample

Collutory containing benzydamine hydrochloride (0.15%, w/v) is a commercial product (Tantum Verde, Aziende Chimiche Riunite Angelini Francesco – A.C.R.A.F. S.p.A., Rome, Italy).

Preparation of standard and sample solutions

Standard stock solutions of BZH, BI, DAP, DABA, BDA, BDAP, and famotidine, all at concentration of 1.0 mg/mL, were prepared in the mobile phase. Collutory (5 mL) samples were mixed with 2 mL of 0.1 M sodium hydroxide and 1.0 mL of internal standard (50 µg/mL) in a 50-mL plastic centrifuge tube (Falcon Plastics, Oxnard, CA). Ethylacetate (3 mL) was added to the samples, and the tubes were shaken for 10 min in a Dubnoff mechanical shaker (150 cycles/min). Separation of the two phases was achieved by centrifugation at 3000 rpm for 10 min, and the organic phase was transferred into a second tube. Fresh ethylacetate (3 mL) was added to the first tube, and the same extraction procedure was repeated twice. The organic phases were collected from the three extractions of the same sample and pooled. The organic phase was evaporated to dryness under a stream of nitrogen. Four hundred microliters of mobile phase were added to the residue. The tube was shaken for two min, and the solution obtained was filtered through a 0.22-µm filter (Millex PTFE, Millipore). Aliquots of 20 µL were injected into the HPLC system.

Method validation

The method was validated according to the ICH requirements (13). It was evaluated for linearity, detection and quantitation limits, precision, accuracy, robustness, selectivity, and specificity.

Linearity, detection and quantitation limits, precision, and accuracy. Appropriate aliquots of stock solutions were transferred into 10-mL volumetric flasks and diluted to volume with mobile phase. The examined concentration ranges were from 70 to 120 µg/mL for BZH, from 10 to 100 µg/mL for DAP, DABA, BDAP; from 10 to 150 µg/mL for BI; and from 10 to 200 µg/mL for BDA. Famotidine was added at a concentration of 50 µg/mL. Then, the solutions were filtered using a 0.22-µm filter (Millex PTFE, Millipore) and injected into the HPLC instrument. Each solution was injected in triplicate. Peak-area ratios (between analyte and internal standard) were plotted versus the respective analyte concentrations.

Limit of detection. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the residual standard deviation of the regression line (σ) of the analytical curve and its slope (S) in accordance with the equations LOD = 3.3(σ/S) and LOQ = 10(σ/S) (18–20).

In order to measure repeatability of the system, six consecutive injections were made using a standard solution containing 80 µg/mL of BZH and 50 µg/mL of the internal standard. The results were expressed as the percent relative standard deviation (%RSD) for the peak-area ratio of the BZH/internal standard ratio and the retention time of BZH.

Ten determinations were performed to establish the intra-day precision. The intra-day precision was evaluated by injecting sample solutions prepared at low, middle, and high concentrations of the analytical curve (70–120 µg/mL) containing 50 µg/mL of internal standard in one day. The inter-day precision was evaluated by injecting the same solutions on three consecutive days. Three determinations for each concentration were performed. Precision was expressed as %RSD for the peak-area ratio of BZH/internal standard and retention time of BZH. The accuracy was calculated as the percentage recovery of a known amount of standard added to the sample. The accuracy of method was evaluated in triplicate using three concentration levels: 80, 100, and 120 µg/mL.

Selectivity. The selectivity of the method was established through a study of retention time, retention factor, separation factor, resolution of all peaks, and the absorption spectra of the eluted peaks.

Table I. Chromatographic Parameters*

Compound	Retention time (t_R, min)	Retention factor (k)	Relative retention (rt_R)
BZH	10.0	13.1	BZH/BZH = 1.0
DAP	14.1	18.8	DAP/BZH = 0.7
DABA	4.8	5.8	DABA/BZH = 2.1
BI	4.4	5.3	BI/BZH = 2.3
BDA	3.0	3.2	BDA/BZH = 3.3
BDAP	12.4	16.5	BDAP/BZH = 0.8

* t_o = 0.71 min.

Specificity. Specificity of the method was tested by observing the potential interferences among the investigated substances and the excipients. No interfering peaks were present in the chromatogram of the placebo sample.

Robustness. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage (19). In order to study the robustness of the proposed method, deliberate modifications in flow rate, wavelength values, buffer pH, and mobile phase composition were made.

Results and Discussion

Method development

In order to develop a simple and efficient HPLC method for quantitative determination of BZH and five of its impurities in collutory formulations, preliminary tests were performed with the objective to select optimum conditions. Different organic modifiers were evaluated (methanol, acetonitrile, tetrahydrofuran, and isopropanol) in different proportions. Tetrahydrofuran and isopropanol did not provide satisfactory chromatographic profiles (overlaps in impurity products peaks and excessive drift), while mixtures of methanol and acetonitrile showed an improvement of the separation of all the analytes and a reduction in the time of analysis. Owing to acid-base characteristic of the compounds herein analyzed, separation is highly dependent on pH, so the effect of this variable was studied. The buffer pH was tested in the range of 9.5–11.5 units and was found that the increase of pH from 9.5 to 10.5 led to an improvement in the peaks symmetry and resolution, while further increase didn't give appreciable improvement. The effects of buffer concentration on analytes retention time was also studied over the range of 5–50 mM and at constant pH. Best results were obtained at 10 mM.

During the optimization phase two RP columns were compared: Gemini C_{18} and XTerra. Gemini columns have been on the market for some years. They are made with high purity HPLC silica, are stable over the pH range of 1–12, and are indicated for retention of basic compounds. Xterra columns are based upon first generation hybrid particles. The presence of 33% fewer residual silanols (after endcapping and bonding) also means that these columns give exceptionally sharp, high-efficiency peaks for basic compounds. Unfortunately, XTerra produced chromatograms with two unresolved pairs of peaks (impurities DABA and BI, and impurities BDA from the internal standard). As results of these experiences, the Gemini RP column, a mobile phase constituted from a mixture of ammonium carbonate buffer (10 mM; pH 10.5), and acetonitrile and methanol in equal proportion as organic modifiers were used. This mobile phase was suitable to obtain an adequate separation of all analytes and the internal standard. Different flow rates were also tested to achieve efficient separation with a satisfactory resolution in a short analysis time. The retention parameters of the investigated substances, which are presented in Table I, indicate the optimal experimental HPLC

conditions. Figures 2 and 3 show the chromatogram obtained after injection of 20 µL of the working standard solution and the chromatogram of the placebo solution after extraction, respectively. Figure 4 shows the representative chromatogram after the injection of the sample solution of collutory after extraction.

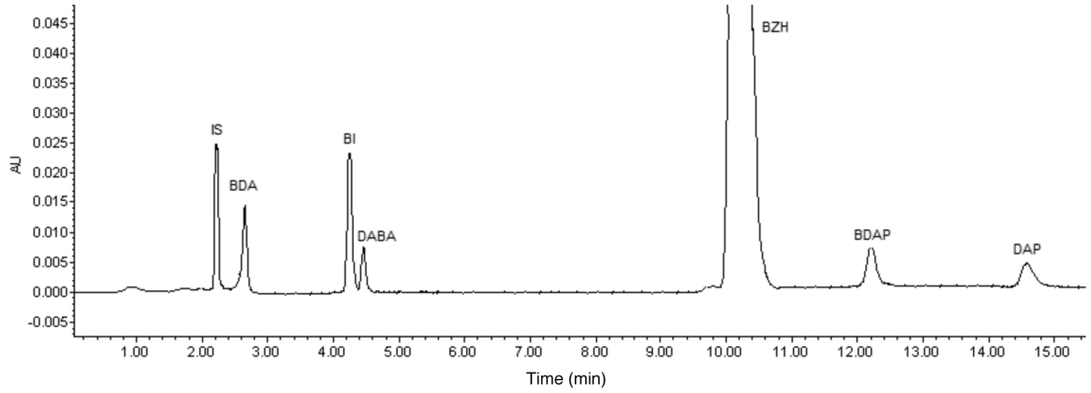

Figure 2. The representative chromatogram of a standard solution. Conditions: Gemini C18 column (250 × 4.6 mm i.d., 5 µm); mobile phase: acetonitrile, methanol, and ammonium carbonate buffer (10 mM; pH 10.5) (37.5:37.5:25, v/v/v), temperature of the column 30°C; flow rate: 1.0 mL/min; UV detection at 218 nm.

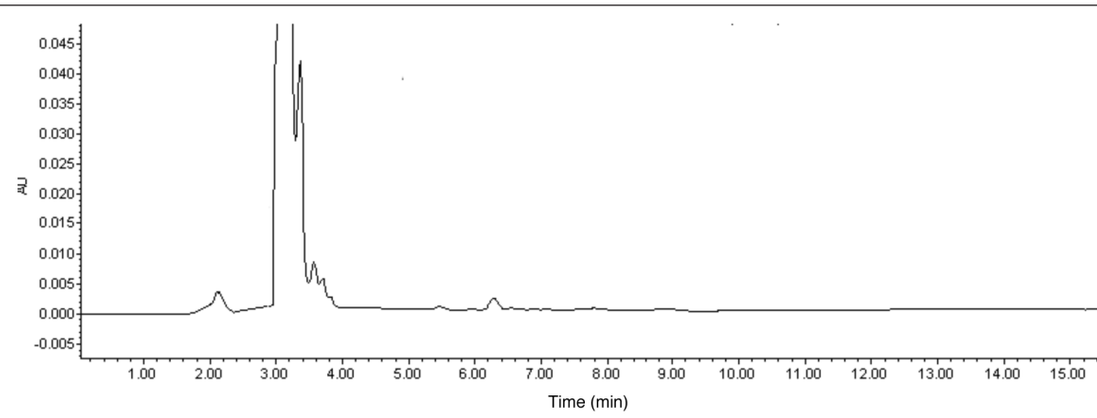

Figure 3. Placebo solution containing excipients of the pharmaceutical formulation after extraction process. Conditions: Gemini C18 column (250 × 4.6 mm i.d., 5 µm); mobile phase: acetonitrile, methanol, and ammonium carbonate buffer (10 mM; pH 10.5) (37.5:37.5:25, v/v/v), temperature of the column 30°C; flow rate: 1.0 mL/min; UV detection at 218 nm.

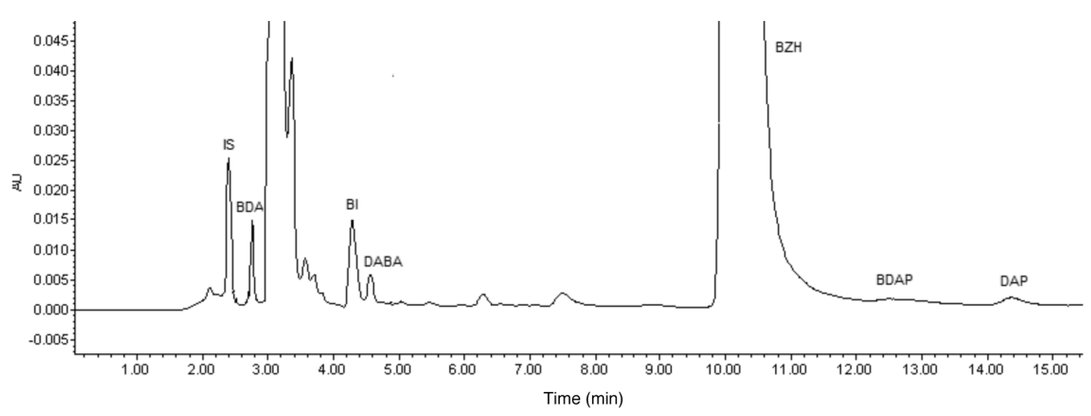

Figure 4. The representative chromatogram of a sample of a commercial collutory. Mobile phase: acetonitrile, methanol, and ammonium carbonate buffer (10 mM; pH 10,5) (37.5:37.5:25, v/v/v); temperature of the column 30°C; flow rate 1.0 mL/min.

Method validation

Analytical curves were obtained by plotting peak-area ratios (analyte-to-internal standard) against the concentrations of the respective analytes. In all cases, straight regression lines with correlation coefficients (*r*) above 0.998 were obtained (21,22). The F-test applied for all calibration curves and data provides conclusive evidence of a linear relationship between concentration and instrumental response (21). The data are summarized in Table II.

The LOD and the LOQ were calculated using analytical curves results (Table II). The LOD and the LOQ values were obtained by injecting 20 µL of standard solutions into the chromatographic system. Injection precision was determined after injecting ten times into the chromatographic system a benzydamine hydrochloride standard solution containing 80 µg/mL. The obtained results, expressed as %RSD, were 0.3% for peak-area ratio and 0.06% for retention time. The values obtained demonstrate that the system is reliable for the analysis. The precision of the method was evaluated by repeatability and intermediate precision determinations. For repeatability, ten sample solutions at 100 µg/mL were analyzed on the same day, and the %RSD obtained was 1.9%. The intermediate precision was achieved by analyzing three different concentrations on three consecutive days. The results, which, are shown in Table III present good agreement. The accuracy of the method was evaluated at three concentration levels. Triplicate determinations were made at each concentration level. The accuracy is expressed as a percentage of standard recovered from sample matrix. The results are shown in Table IV. For robustness determination, changes in flow rate, wavelength values, buffer pH, and composition of mobile phase were evaluated (Table V).

Table II. Regression Analysis Data for BZH and Its Impurities

Statistical parameters	BZH	DAP	DABA	BI	BDA	BDAP
Concentration range (µg/mL)	70–120	10–100	10–100	10–150	10–200	10–100
Intercept	21050	16829	21689	29308	55630	31354
Slope	44657	11265	9410.9	9853.6	6683.3	10595
Correlation coefficient (*r*)*	0.9987	0.9981	0.9990	0.9991	0.9986	0.9988
S_a*	0.034	0.015	0.009	0.009	0.006	0.011
S_b*	0.002	0.002	0.001	0.002	0.001	0.002
LOD (µg/mL)*	1.15 5.42	2.42 8.24	2.12 9.32	1.23 7.78	1.31 7.96	1.84 9.86
LOQ (µg/mL)*	785.44	864.18	678.72	567.84	688.41	569.91
F*						

* LOD = limit of detection; LOQ = limit of quantitation; *r* = correlation coefficient; S_a, S_b = standard deviations of the intercept and slope; F-test tabulated (0.05) = 7.71.

Method application

The method was applied to assay benzydamine and its impurities in a pharmaceutical formulation. Analyzing the collutory, impurities DAP, DABA, BDA, and BI were found, while BDAP wasn't present in the sample. The content of benzydamine was 99.8% (± 0.2), while the content of impurities DAP, DABA, BDA, and BI was lower of 0.2%.

Conclusions

This paper describes an isocratic RP-HPLC–UV–DAD method that allows for reliable quality control of BZH and impurities in formulations with complex composition. The method can be applied in typical analytical laboratories and does not require sophisticated instrumentation. Total time of the analysis was about 15 min. The applied method can be used in the quality control and purity testing of the collutory as sensitive, precise, and accurate.

Table III. Intra- and Inter-day Precision of the Proposed RP-HPLC Method for BZH Quantitative Determination

	80 µg/mL	100 µg/mL	120 µg/mL
Intra-day (*n* = 3) % RSD	2.11	0.29	1.87
Inter-day (*n* = 9) % RSD	0.64	1.38	0.87

Table IV. Recovery of a Standard Solution of BZH Added to the Sample and Analyzed Using the Proposed HPLC Method

Standard added to commercial sample (µg/mL)*	Standard found (µg/mL)	Recovery (%)†
40.00	39.30	98.2
50.00	51.40	102.8
60.00	60.50	100.8

* Commercial sample (BZH collutory).
† Average of three determinations.

Table V. Robustness Data for the Assay of BZH

Condition	Method variable	Standard (BZH peak) Symmetry factor	Column efficiency	Capacity factor (k^1)
Limit		*≤ 2.0*	*≥ 4000*	*6.0–10.0*
1		0.99	6793	6.0
2	Wavelength	0.99	6755	6.1
3		0.99	6738	6.1
4	Flow rate	0.98	6690	6.2
5		0.99	6711	6.0
6	Mobile phase composition	0.98	6925	6.2
7		0.99	6899	6.2
8	Buffer pH	0.99	6692	6.1
9		0.98	6712	6.4
10		0.99	6800	6.0
Mean		0.99	6672	6.1
% RSD		0.46	1.16	1.94

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