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Quantitation of Metronidazole in Pharmaceutical Suspension Using High Performance Liquid Chromatographic Method

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Abstract.- A simple and rapid HPLC (High performance liquid chromatography) assay procedure has been developed and validated for the quantitative determination of metronidazole benzoate for raw material and commercially available suspensions. The HPLC conditions used were, column: C18, (0.5µm particle size, 150×4.6mm); detection: UV at 254 nm; injection volume: 20 µl; mobile phase: methanol and de-ionized water (64:40, v/v); isocratic elution under ambient temperature at flow rate of 1.0 ml min^{-1}. The procedure separated metronidazole benzoate, in an overall analysis time of about 10 minutes with metronidazole benzoate eluting at about 7.7 minutes. The injection repeatability was 0.05%, the intraday and interday repeatability were 0.59 and 0.91%, respectively. The procedure provided a linear response over the concentration range 10–1000 µgml^{-1} (r^2 = 1.0000) with the limits of detection and quantitation 0.0158 and 10 µg ml^{-1}, respectively. The results show no detectable hydrolysis of metronidazole benzoate in 10 hours.

Key words: Metronidazole benzoate, Reverse-phase HPLC, suspension, methanol, precision

INTRODUCTION

Metronidazole (*1-β-hydroxyethyl-2-methyl-5-nitroimidazole*) belongs to nitroimidazole group that is a relatively inexpensive, highly versatile drug with clinical efficacy against broad spectrum anaerobic bacteria. The uses of metronidazole for antiprotozoal therapy have been reviewed extensively **(**Freeman *et al.*, 1997; Johnson, 1993; Stanley, 2003; Nash, 2001**)**. It is clinically effective in trichomoniasis, amebiasis, and giardiasis, as well as in a variety of infections caused by obligate anaerobic bacteria, including *Bacteroides, Clostridium, Fusobacterium, Peptococcus, Peptostreptococcus, Eubacterium,* and microaerophilic bacteria such as *Helicobacter* and *Campylobacter* spp. Metronidazole antimicrobial properties are thought to derive from the formation of toxic-free radicals by intracellular reduction.

Both parenteral and enteral routes have been used to administer metronidazole. Nevertheless, the need for additional dosage forms have been warranted for patients who require a different dosage form than what is commercially available *(e.g.,* patients who are incapable of swallowing tablets or capsules or pediatric patients who are incapable of swallowing available dosage forms). Recently a number of oral suspensions of metronidazole benzoate have been marketed.

For estimation of metronidazole, several methods have been used *i.e.,* spectrophotometry *e.g.* metronidazole and hycanthone (Bahia and Moussa, 1982), metronidazole and nalidixic acid (Parimoo *et al*., 1996), metronidazole and diloxanide furoate (Indravadan *et al*., 1997); thin layer chromatography; Supercritical fluid chromatography *e.g.* metronidazole and nalidixic acid (Viddesh *et al*., 1998), electrochemistry, and voltammetry *e.g.* metronidazole (Ozkan *et al*., 1998), high-performance liquid chromatography (HPLC) e.g. reverse phase HPLC has been used for the simultaneous determination of metronidazole and miconazole in pharmaceutical dosage forms. Chromatography was carried out on a C18 reversed-phase column, using a mixture of methanol–water (40+60, v/v) as mobile phase, at a flow rate of 1.0 ml min^{-1}. Sulfamethoxazole was used as an internal standard and detection was performed using a diode array detector at 254 nm. The method produced linear responses in the concentration ranges 10–70 and 1–20 µg ml^{-1} with detection limits 0.33 and 0.27 µg ml^{-1} for metronidazole and miconazole, respectively. This procedure was found to be convenient and reproducible for analysis of these

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drugs in ovule dosage forms (Cemal *et al.,* 2003). Similarly simultaneous determination of metronidazole benzoate (MB), diloxanide furoate (DF), methyl paraben (MPn) and propyl paraben (PPn) in suspension was studied (Mishal and Sober*,* 2005) in which mobile phase, a phosphate buffer-acetonitrile mixture (70:30, v/v) adjusted to pH of 2.5 at a flow rate of 2.0 mL min^{-1}. In addition simultaneous estimation of amoxicillin and metronidazole at single wavelength (254 nm) using C_{18} column with buffered mobile phase (pH 4.0) and was not affected by excipients (Naser *et al.,* 2007). Metronidazole benzoate was studied by Daniel *et al*. *(*2005) who used UV at 271 nm; injection volume: 20 µl; mobile phase, acetonitrile and 0.1% glacial acetic acid in monobasic potassium phosphate (0.01 M) (40:60 v/v); isocratic elution under ambient temperature at 2.0 ml min^{-1}. The injection repeatability was 0.03%, and the intraday and interday repeatability were 0.4 and 0.7%, respectively. The procedure provided a linear response over the concentration range 0.2–800 µg ml^{-1} (r = 1.0000) with the limits of detection and quantitation 0.03 and 0.2 µg ml^{-1}, respectively. To our knowledge very few reports are available for metronidazole benzoate and these are not related to suspension.

In the present study we report a simple, rapid and accurate HPLC assay procedure that can quantitate metronidazole benzoate in raw material as well as in suspension and this method is unaffected by excipients present in suspension. Mean recovery percentage of metronidazole benzoate by this procedure is higher than previously reported.

MATERIALS AND METHODS

Chemicals

Metronidazole Benzoate Standard (Kindly provided by Sanofi-Aventis Pakistan Limited, Karachi), Metronidazole Benzoate Raw material (Kindly provided by Sanofi-Aventis Pakistan Limited, Karachi), HPLC Grade Methanol (Merck Darmstadt, Germany), De-ionized Water, Metronidazole Suspension: Flagyl 200mg/5ml (Aventis Ltd Karachi), Phosphoric Acid (Merck Darmstadt, Germany).

Preparation of standard solutions

Metronidazole benzoate (643 mg) reference standard equivalent to 400 mg of metronidazole was weighed accurately on an analytical balance and was taken in a 200 ml volumetric flask. It was dissolved completely with methanol and the volume was made up to the mark with the same solvent. Standard solutions of different concentration *i.e.,* 50, 100, 200, 500 and 1000 µgml^{-1} were prepared. These solutions were filtered through 0.45 micron membrane filter. Standard graph was plotted concentration against area under curve by High performance liquid chromatography (HPLC).

Preparation of sample solutions

Five ml of the metronidazole suspension was taken in 200 ml volumetric flask with the help of pipette. Added 150 ml of methanol and dissolved completely by ultrasonic bath. The volume was made up to the mark with the same solvent. 2.5 ml of the above solution was taken in another 100 ml volumetric flask, added de-ionized water up to the mark and mixed well. Filtered through 0.45 micron membrane filter. The concentration of the above solution is 50 µgml^{-1}.

Preparation of mobile phase for HPLC

Methanol (HPLC grade) and de-ionized water were mixed in the ratio of 650:400 ml and the pH was adjusted to 2.5 by phosphoric Acid. The mobile phase was filtered through 0.45 µ membrane filter by vacuum filtration unit and then degassed for 15 minutes by ultrasonic bath.

HPLC analytical procedure

An aliquot of 20 µl sample was injected on HPLC (Shimadzu LC-10ATvp) column (C18, 0.5µm particle size, 150×4.6mm) having Flow Rate 1 ml/minute at ambient temperature using detector (UV 254 nm; chart speed 5 mm/minute; attenuation 8 (adjustable) and sensitivity 0.01). Samples were used in five replicate. Major peak response of metronidazole benzoate in assay preparation and standard preparation were recorded in the chromatogram.

Validation of analytical method

The analytical method for measurement of

the drug was validated for specificity, linearity (evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares), accuracy (assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range, 3 concentrations 3 replicates each of the total analytical procedure reported as percent recovery by the assay of known added amount of analyte in the sample), precision (assessed by a minimum of 6 determinations at 100 percent of the test concentration) and sensitivity.

Calculations

The percentage of metronidazole benzoate was calculated by the formula given below.

$$\% \text{ age of metronidazole benzoate as per labeled amount} = SPA/STA*C_2/C_1*F$$

where SPA is the peak area of the metronidazole benzoate in assay preparation, STA is the peak area of the metronidazole benzoate in standard preparation, C_2 is the concentration in $\mu g ml^{-1}$ of metronidazole benzoate in standard preparation, C_1 is the concentration in $\mu g ml^{-1}$ of metronidazole benzoate in assay preparation and F is the exact percentage purity of the metronidazole benzoate reference standard.

RESULTS AND DISCUSSION

HPLC procedure development using metronidazole raw material

After the preparation of standard curve (Fig.1) for metronidazole benzoate (MB) reference material, raw material was analyzed by the proposed method at different concentrations and compared, one of the comparisons is shown in Figure 2. In order to identify the impurities under isocratic conditions, the mixtures of methanol with water in different combinations were assayed as the mobile phase using C18 packing as stationary phase. Binary mixture of methanol-/water in proportion of 65:40 (v/v), proved to be better than the mixture of acetonitrile-water for the separation since the chromatographic peaks were better defined and resolved, and almost free from tailing. Among several flow rates tested (0.5-2 ml min^{-1}), the rate of 1.0 ml min^{-1} was the best with respect to location and resolution of analytical peaks.

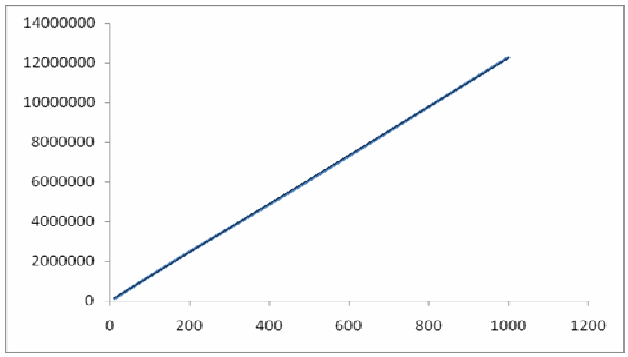

Fig. 1. Standard graph between metronidazole concentration (µg/ml) on X-axis While chromatographic area (mV) on Y-axis

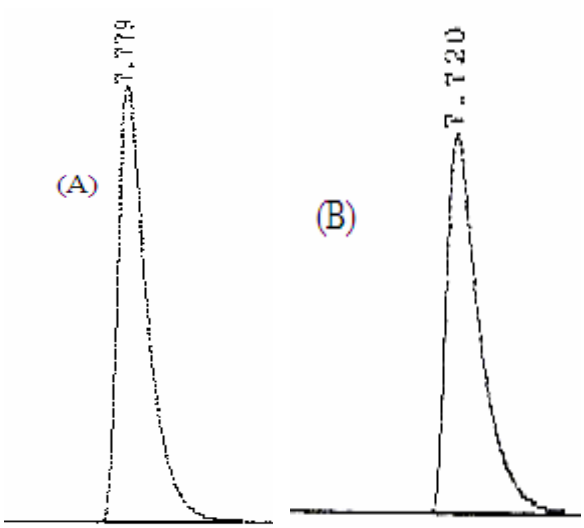

Fig 2. Chromatograms showing peak of Metronidazole as metronidazole benzoate in standard solution containing 200 µg ml^{-1} (A) and its raw material containing 250µg ml^{-1} (B) using mobile phase, methanol-water (65:40; v/v), flow rate 1.0 ml min^{-1} and detection wavelength 254 nm.

Retention time of metronidazole in standard solution was 7.751±0.00025 (0.016) and in raw material 7.755±0.00089 (0.029) which indicate consistency of retention time using proposed mobile phase ratios and from the Table I it is clear that the percentage purities of the raw material of metronidazole benzoate at different concentrations are within the BP range of (99-101%).

Table I.- Characteristics of linear regression analysis of metronidazole benzoate (MB)

Parameters	Values	Comments
Linearity range (μg ml^{-1})	10-1000	Intercept was not significantly different from zero (96% CL)
Slope	12248	Area under curve (mV)
Intercept	25106	Area under curve (mV)
Correlation co-efficient	1.000	
Injection repeatability	0.05%	n=6
Intraday repeatability[a]	0.59%	n=4
Interday repeatability[b]	0.91%	n=4
Solution stability	≥ 10 hrs	Standard solution of metronidazole benzoate in mobile phase was stable for at least 10 h
Limit of quantitation (LOQ)	10 μg ml^{-1}	Precision 1.2%
Limit of detection (LOD)	0.0158 μg ml^{-1}	
Mean Recovery % in Standard reference	100.01	
Mean Recovery % in Raw material	100.09	

[a] Mean values represent three different sample standards for each concentration.
[b] Interday reproducibility was determined from three different runs for each concentration over a 2-weeks period.

The injection (system) precision was determined by performing six replicate injections of the standard solution (nominally, 200 μg ml^{-1}). The characteristics of regression equations and the working concentrations are given in Table I. The limit of detection (LOD) and limit of quantitation (LOQ) of the procedure are also shown in Table II, which were calculated according to the 3s /m and 10s /m criterions, respectively, where s, is the standard deviation of the peak area ratios (four injections) of the sample and m is the slope of the corresponding calibration curve (Swartz and Krull, 1997). The intra- and inter-day variations of the method were determined on commercial products using three replicate injections of four different concentrations, which were prepared and analysed on the same day and on three different days over a period of two weeks, respectively. The R.S.D. values were 0.59 and 0.91%, respectively (Table II). The assay results of metronidazole benzoate in standard solutions (nominally, 200 μg ml^{-1}), prepared and assayed on each of three consecutive days averaged 97.69% and the precision (R.S.D.) was 0.91%. This indicates a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs. Sample solutions analysed after 48 h did not show any appreciable change in assay values.

Table II.- Names and dosage form of commercially used suspensions of metronidazole benzoate.

No.	Name of preparation	Composition (Each 5ml contains)	Manufacturer
1	Flagyl suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Sanofi-Aventis Limited, Karachi. Pakistan
2	Diazol suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Atco Limited, Karachi, Pakistan
3	Menidazole suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Werrick Limited, Islamabad, Pakistan
4	Metrozil suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Siza International Limited, Lahore, Pakistan
5	Metrozine suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Searle Limited, Karachi, Pakistan
6	Protomet suspension	Metronidazole benzoate eq. to metronidazole base =200mg	Geofman Limited, Karachi, Pakistan

Assay in pharmaceutical dosage forms

On the basis of above results, the proposed method was applied for the determination of metronidazole benzoate in commercial suspensions

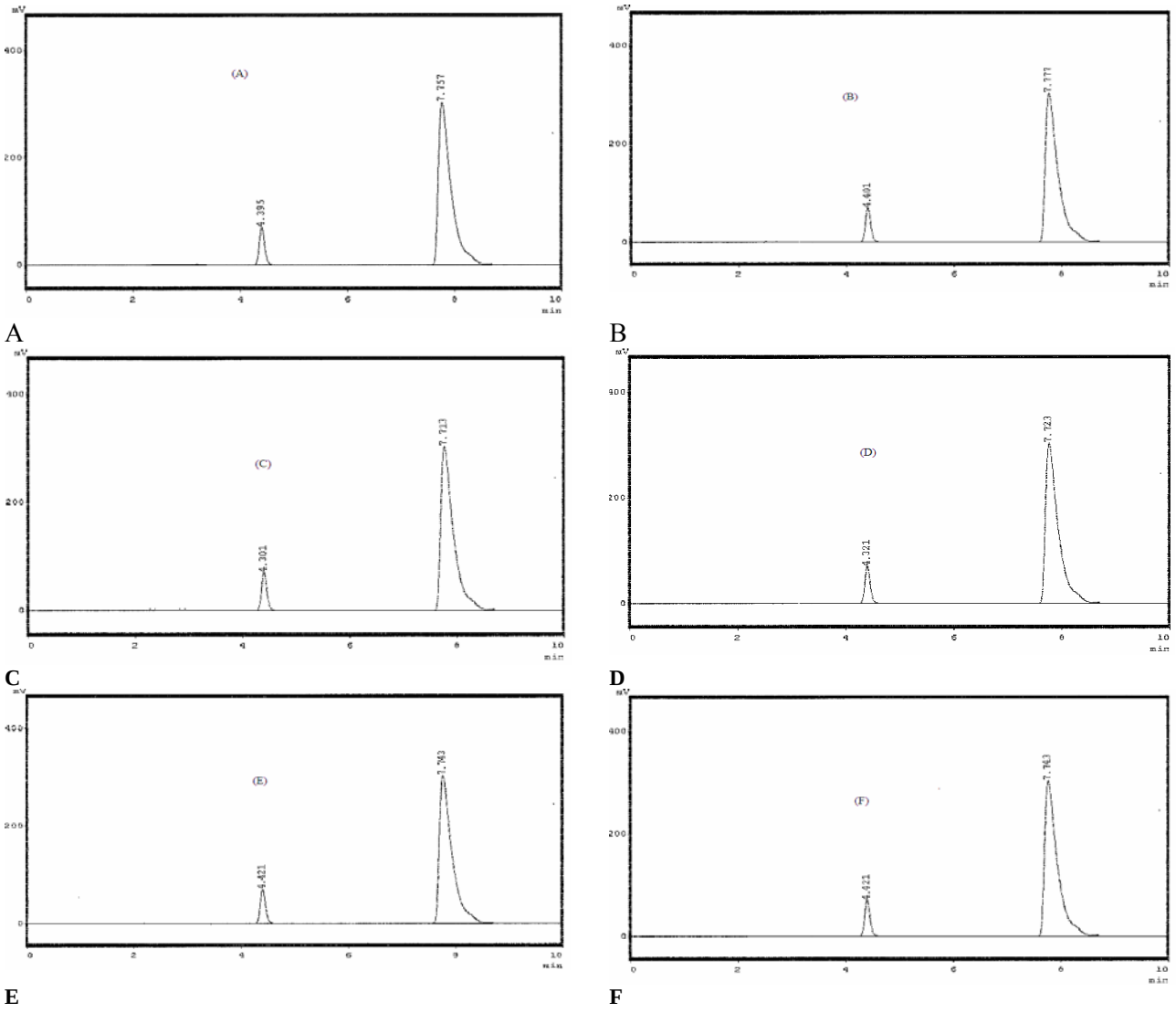

Fig. 3. HPLC Chromatograms of Flagyl MB suspension (A), Diazol (B), Menidazole (C), Metrozil (D), Metrozine (E) and Protomet suspension (F)

(Table II) which comprised various excipients [sucrose, ethanol, special magnesium silico aluminate (veegum), sodium sacccharine dihydrate, sodium dihydrogen phosphate dihydrate, methylparahydroxybenzoate, concentrate lemon essence, deterpenated orange essence, propylpara-hydroxybenzoate]. Figure 2 shows typical chromatograms obtained for the analysis of MB in suspensions. The differences between the amount claimed and those measured were very low and the RSD values were within the acceptable windows mentioned by pharmacopoeias. When various quantities of suspensions (Equivalent to 400 µg ml^{-1} of metronidazole benzoate) were assayed by HPLC, the mean values of Flagyl suspension was 409.80, Diazol suspension (405.60 µg ml^{-1}), Menidazole (415.00 µg ml^{-1}), Metrozil suspension (402.80 µg ml^{-1}), Metrozine suspension (410.92 µg ml^{-1}), Protomet suspension (408.80 µg ml^{-1}).

The recovery of the procedure was carried out by spiking the already analyzed standard samples of metronidazole benzoate (MB). Percentage recovery by Flagyl suspension was102.27, Diazol 101.40, Menidazole 103.75, Metrozil suspension100.70,

Metrozine suspension 102.73 and Protomet suspension102.20 respectively (n=6), indicating that the results are accurate, precise and there is no interference from the common excipients used in the suspension formulation.

Accuracy was assessed using Flagyl in minimum of nine determinations over not less than three concentration levels (60, 250 and 700 μg ml^{-1}) covering the specified range (3 concentrations/3 replicates each of the total analytical procedure). Statistical analysis showed concentration 100.37±1.08 (1.04) that the precision (R.S.D.) was 1.71%.

CONCLUSIONS

It can be concluded that the proposed method is sufficiently sensitive and reproducible in the analysis of metronidazole benzoate in raw material and in pharmaceutical suspension forms within a short analysis time (<8 min). The proposed HPLC method was validated by evaluation of the validation parameters e.g. linearity range, solution stability, relative standard deviation of slope and intercept, correlation coefficient, within- and between-day reproducibility, LOD, LOQ values, for this technique were obtained.

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REFERENCES

BAHIA, A. AND MOUSSA, 1982. Colorimetric determination of parasiticides hycanthone and metronidazole. *Int. J. Pharm*., **10**: 199-207.

CEMAL AKAY, SIBEL A., OZKAN, ZUHRE, SENTURK, AND SEMSETTIN CEVHERO LU, 2003. Simultaneous determination of metronidazole and miconazole in pharmaceutical dosage forms by RP-HPLC. *II Farmaco*, **57:** 953-957.

DANIEL, K., BEMPONG, G., MANNING, R., MIRZA, T. AND BHATTACHARYYA, L., 2005. A stability-indicating HPLC assay for metronidazole benzoate. *J. Pharmaceut. Biomed. Anal.,* **38**:776-780.

FREEMAN, C.D., KLUTMAN, N.E. AND LAMP, K.C., 1997. Metronidazole: A therapeutic review and update. *Drugs,* **54**: 679-708.

INDRAVADAN, C., RAMAN, B.B., SUNDARESAN, M. AND BHAGWAT, A.M., 1997. Separation and estimation of diloxanide furoate and metronidazole in solid dosage form using packed column supercritical fluid chromatography. *Anal. Chim. Acta*, **354**: 123-128.

JOHNSON, P.J., 1993. Metronidazole and drug resistance. *Parasitol. Today*, **9**:183-186.

MISHAL, A. AND SOBER, D., 2005. Stability indicating Reversed-phase liquid chromatographic determination of metronidazole benzoate and diloxanide furoate as bulk drug and in suspension dosage form. *J. pharm. Biomed. Anal.,* **39:** 819-823**.**

NASER, T., VARSHOSAZ, J., DORKOOSH, F. AND ZARGARZADEH, M.R., 2007. Development and validation of a simple HPLC method for simultaneous in vitro determination of amoxicillin & metronidazole at single wavelength. *J. Pharm. Biomed. Anal.*, **43**: 325-329.

NASH, T. E., 2001. Treatment of *Giardia lamblia* infections. *Pediatr. Infect. Dis. J*., **20**:193-195.

OZKAN, S.A., OZKAN, Y. AND SENTURK, Z., 1998. Electrochemical reduction of metronidazole at activated glassy carbon electrode and its determination in pharmaceutical dosage forms. *J. Pharm. Biomed. Anal*., **17**: 299-305.

PARIMOO, P., PRASAD, C.V. AND VINEETH, R., 1996. Simultaneous quantitative determination of metronidazole and nalidixic acid in tablets by difference spectroscopy. *J. Pharm. Biomed. Anal*., **14**: 389-93.

STANLEY, S.L., 2003. Amoebiasis. *Lancet*, **361**: 1025-1034.

SWARTZ, M.E. AND KRULL, I.S., 1997. *Analytical method development and validation*, Marcel Dekker, New York, pp. 61-62.

VIDDESH, R., DHORDA, B. U. J. AND SUNDARESAN, M., 1998. Simultaneous estimation of nalidixic acid and metronidazole in dosage forms using packed column supercritical fluid chromatography. *Anal. Chim. Acta*, **376**: 221-225.

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