ELSEVIER

Journal of Chromatography A, 750 (1996) 397–402

JOURNAL OF CHROMATOGRAPHY A

Short communication

Quantification of cyclamate and cyclohexylamine in urine samples using high-performance liquid chromatography with trinitrobenzenesulfonic acid pre-column derivatization

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Abstract

An HPLC isocratic method with pre-column derivatization and UV detection for the quantification of cyclamate and cyclohexylamine in urine samples is described. The method requires very little sample preparation. Free cyclohexylamine is analysed in a first run and subsequently cyclamate is analysed as cyclohexylamine, after the simple process of oxidation of the sample by means of hydrogen peroxide. Cycloheptylamine is used as internal standard. Trinitrobenzenesulfonic acid (TNBS) appears to be a good reagent for the pre-column derivatization. The time per run is 15 min; the coefficients of variation of the assays range from 1.1 to 5.5%; the limits of detection are 0.09 and 0.11 ppm for cyclohexylamine and cyclamate anion, respectively. The system described has always performed efficiently, with a high degree of stability, in daily routine work.

Keywords: Cyclamate; Cyclohexylamine; Trinitrobenzenesulfonic acid; Derivatization, LC

1. Introduction

Cyclamic acid, cyclohexanesulfamic acid [CAS No. 100-88-9], and its salts, cyclamates [CAS No. 139-05-09], are used either as dietary sweeteners (sodium salt) or as sweeteners for low sodium diets (calcium salt). Many countries use these products as food additives. In Europe, cyclamic acid is also known as the authorized food additive E 952, in accordance with the European Parliament Directive 94/35/CE of 30 June 1994.

Cyclamate is excreted unchanged in urine and faeces [1,2]. However, providing the cyclamate or its salts have formed part of their diet for a sufficient period of time some people, called 'converters', transform cyclamate to cyclohexylamine by means of a microorganism acting in their colon and rectum [3]. There are inter-individual differences among people with regard to the ability to metabolise cyclamate: over 70% of the human population are unable to metabolise cyclamate, while only 3 to 5% of the population metabolise more than 20% of their daily intake. Moreover, there is also considerable intra-individual variability relating to intestinal transit rate, among other factors. Finally, after a single dose, the plasma half-life of cyclamate and cyclohexylamine is 8 and 4 h, respectively [4].

In 1969, the discovery of bladder tumours in rats fed high concentrations of a cyclamate–saccharine mixture (10:1), supplemented with cyclohexylamine, led to the banning of cyclamate in the USA and other countries [5]. However, a number of more recent studies suggest that neither cyclamate nor cyclohexylamine causes cancer in humans or experimental

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0021-9673/96/$15.00
PII S0021-9673(96)00529-8

animals such as rats, mice, monkeys, dogs or hamsters [6].

Even if questions concerning the carcinogenic potential of cyclamate and cyclohexylamine appear to have been resolved, other toxicological issues nevertheless remain [7]. An important consequence of ingesting cyclohexylamine is its sympathomimetic activity, although this has not been observed in humans [8]. A laxative effect of sodium and calcium cyclamate has also been recorded in the case of very high doses [9]. Moreover, some studies have reported that oral administration of cyclohexylamine produces testicular disfunctions in rats, but this effect was not observed in other experimental animals (mice), or was reversible (dogs) [10].

The toxicity of a substance may be measured by its teratogenicity in experimental animals. In fact, neither cyclamate nor cyclohexylamine have displayed toxic effects on foetuses after its administration to various experimental animals during pregnancy. Similarly, mutagenic effects have not been observed in the majority of cells (plant cells, mammalian somatic cells, mammalian germ cells, microorganisms). The foregoing as a whole questions the allegedly mutagenic effect of cyclamate and its metabolites in humans [11].

Different methods have been described regarding the analysis of cyclamate in food (confectionary, sirups, jams, dietary products), beverages or pharmaceutical preparations. Among other methods gas chromatography [12], spectrophotometry [13], flow injection analysis [14], isothacophoresis [15] and liquid chromatography [16–22] had been used. Nevertheless, to date no attention has been paid to the analysis of cyclamate in body fluids.

In the present work we report a chromatographic method, based on a 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) derivative of the amine functional group in cyclohexylamine, for the analysis of cyclamate and cyclohexylamine in urine samples. Cyclohexylamine is obtained from the oxidation of cyclamate by means of hydrogen peroxide. Derivatization is followed by reversed phase HPLC with UV detection. In order to ascertain the cyclamate content of a urine sample two analyses are necessary; namely, the crude urine cyclohexylamine content and the oxidized urine cyclohexylamine content. The difference between the molar content in the oxidized sample and the crude sample is the cyclamate molar content in the urine. No special preparation of the urine samples was needed and no interferences were observed. We found TNBS to be a good reagent for cyclohexylamine. Other amines and amino acids were studied by using their TNBS derivatives [23,24].

2. Experimental

2.1. Apparatus

A Kontron 414 pump (Kontron Instruments, Milan, Italy), a Kontron 465 automatic injector, a Kontron 720 LC UV–Vis absorbance detector and a Merck-Hitachi D-2500 integrator (Merck, Darmstadt, Germany) were assembled as an isocratic chromatograph. The column used was a Spherisorb ODS2, 5 μm, 150×4.6 mm (Teknokroma, Sant Cugat, Spain), operated at room temperature. Standard 1.5 ml polypropylene Eppendorf tubes and a Hettich Mikroliter 12 500g centrifuge (Hettich, Tuttlingen, Germany) were used to centrifuge the samples prior to amine derivatization, and a Selecta Them Block (J.P. Selecta, Abrera, Spain) aluminum-heated block was used as a reactor.

2.2. Reagents

All chemicals used were reagent grade. Acetonitrile gradient grade and phosphoric acid were obtained from Carlo Erba Reagenti, Milan, Italy, monoammonium phosphate was purchased from Merck, Darmstadt, Germany, and 1 M 2,4,6-trinitrobenzene-1-sulfonic acid in water was purchased from Fluka, Buchs, Switzerland. The eluent consisted of acetonitrile–0.01 M monoammonium phosphate (60:40, v/v), whose pH was adjusted to 3.5 with a few drops of 4 M phosphoric acid and filtered through a 0.45 μm PTFE filter. Next, both liquids were mixed in a bottle and degassed using a helium flow.

The cyclohexylamine and cycloheptylamine used to prepare the calibration solutions, sodium cyclamate and the TNBS, were purchased from Fluka. Amino acid standard solution (A9906) and urea were obtained from Sigma, St. Louis, MO, USA.

2.3. Samples

Urine samples were aliquots of total 24-h urine collected from two sources. One source was a set of sixteen workers at a cyclamate factory near Barcelona. The other source was a hospital and consisted of 364 samples of daily urine, including blank samples together with other random samples from inhabitants of Barcelona. The samples were kept frozen (−20°C) until some minutes before the analysis.

2.4. Sample preparation

Approximately 1 ml of urine was poured into a 1.5 ml standard polypropylene Eppendorf tube and it was centrifuged at 12 500*g*. A 280 μl aliquot was taken from the supernatant and transferred into a 5 ml glass test tube. 20.0 μl of a cycloheptylamine aqueous solution of known concentration (60 ppm) were added and mixed with a vortex mixer. Next, 100.0 μl 1 *M* of sodium bicarbonate were added, mixed and 100.0 μl 1 *M* of TNBS were poured into the above tube and the resulting solution was mixed again. Some bubbles were observed at this stage. 40.0 μl of 2 *M* sodium hydroxide were added, mixed again and allowed to react for an hour at room temperature. 50.0 μl of 25% (v/v) hydrochloric acid were added to stop the reaction, well shaken and finally 400.0 μl of 96% (v/v) ethanol were added. The solution thus obtained was finally transferred into an automatic injector vial and sealed with a septum and a screw plug.

A similar procedure was followed when oxidized urine was derivatized. Into a 10 ml Pyrex screw-capped glass tube, 2 ml of crude urine, 1 ml of 30% hydrogen peroxide and 0.30 ml of 37% hydrochloric acid were poured and stirred together. Next, the tube was capped, warmed up and kept at 100°C for 1 h. Once the tube had cooled to room temperature, it was further cooled in a fridge to 4°C. After this, the tube was allowed to reach room temperature again and then five drops of 4-nitrophenol pH indicator (0.08 g 4-nitrophenol in 100 ml deionised water) were added and the solution was neutralized with drops of 40% (w/v) aqueous sodium hydroxide until it turned yellow. Finally, the solution was poured into a 5 ml volumetric flask and made level using deionised water.

2.5. HPLC conditions

The eluent was pumped at a rate of 1.0 ml/min and the column effluent was monitored at 335 nm, 0.500 AUFS. Duplicates of ten urine samples were derivatized and 200 μl of each derivatized sample were injected. The readings for the duplicate injections were very close (less than 2% differences in R.S.D.) and therefore, in view of the large quantity of samples to process, only one derivatization per sample was performed in the case of the other samples.

2.6. Standard curves

A six-point calibration was made up where, on the one hand, the concentration of cyclohexylamine in deionized water ranged between 0.3 and 30 mg/l but, on the other hand, the cycloheptylamine concentration remained constant: 6 mg/l at each point. To this end, a large amount of 6 ppm aqueous cycloheptylamine had been previously prepared and this was used as a solvent. The calculations were performed by the internal standard method.

3. Results and discussion

The chromatograms obtained for the samples showed a major front peak before the two peaks under discussion, namely cyclohexylamine and cycloheptylamine (I.S.), see Fig. 1. The retention times were approximately 10.0 and 13.5 min, respectively. The match factor for cyclohexylamine with regard to cycloheptylamine was found to be 1.05. There were no interferences with amino acids, aromatic amines or urea and the resolution was complete. When different amounts of amino acid physiological standard for amino acid analyser and urea were added to the urine samples, no differences were observed with regard to the results obtained from the crude samples.

When blank urine from people without cyclamate in their diet was processed, no peaks appeared in the cyclohexylamine and cycloheptylamine region of the

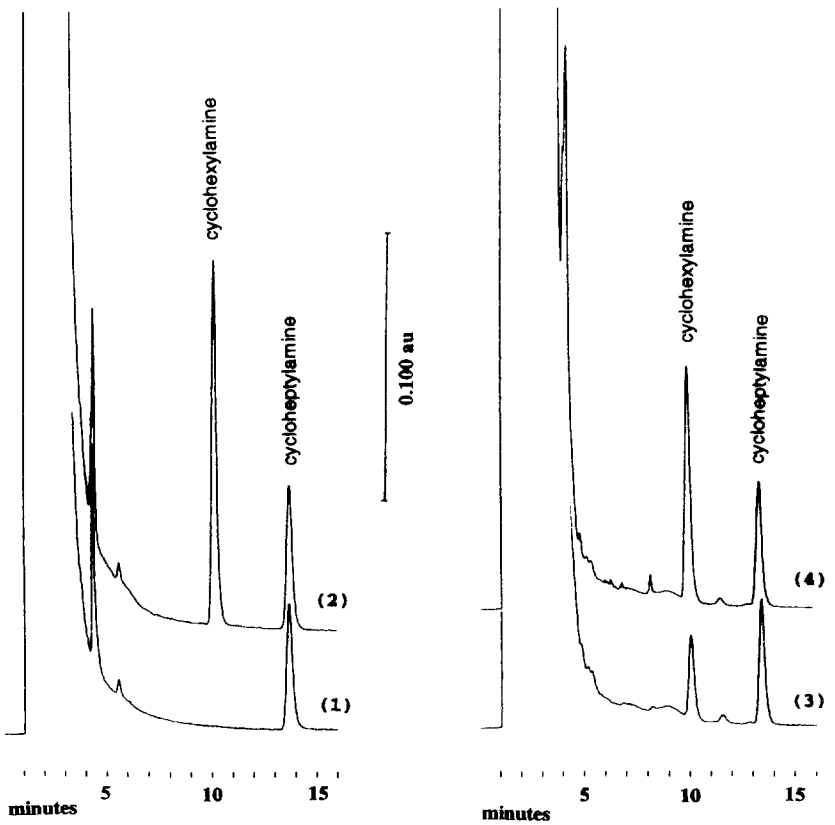

Fig. 1. Chromatograms of N-trinitrophenyl derivatives of cyclohexylamine and cycloheptylamine (I.S.). (See Experimental section for conditions). (1) blank urine; (2) aqueous solution containing 12.0 and 5.0 ppm of cyclohexylamine and cycloheptylamine respectively; (3) crude urine from a 'converter' person showing 1.90 ppm of cyclohexylamine; (4) the previous sample after oxidation, displaying 7.32 ppm of cyclohexylamine.

chromatogram. The levels of free cyclohexylamine in the random population tested ranged between zero and an exceptional value of 49.4 ppm (some people were found to be converters). The levels of cyclamate calculated from the cyclohexylamine found after urine oxidation ranged between lower than the detection limit to an unusual reading of 88.3 ppm of cyclamate anion. The mean values were 1.0 ppm of free cyclohexylamine and 5.17 ppm of cyclamate anion. These results contrasted with the others from target people (workers from a cyclamate factory), where free cyclohexylamine in urine ranged between 0.17 and 6.8 ppm, and the cyclamate anion concentration in urine was found to range between 1.15 and 94.77 ppm. The mean values were 1.84 and 25.7 ppm for free cyclohexylamine and cyclamate anion, respectively, such values being significantly higher than those in random population.

3.1. Validation

The assays performed on the method in order to validate it were the following. The accuracy was calculated as the recovery of an amount of reagent previously added to a blank urine sample, which was then fully processed as if it were a normal sample. Cyclohexylamine was added to two aliquots of this blank urine sample to reach a final concentration of 3.0 and 30.0 ppm, respectively and subsequent analyses showed recovery rates of 99.3 and 96.7%, respectively. On the other hand, when cyclamate was added to reach a final concentration of 2.0 and 20.8 ppm, subsequent analysis revealed recovery rates of 98.7 and 95.3%, respectively, after oxidation into cyclohexylamine (Table 1). These determinations were made as a percentage in relation to the results obtained regarding aqueous reference standards at

Table 1
Recovery of cyclohexylamine and cyclamate added to blank urine ($n=5$)

Cyclohexylamine added (ppm)	Cyclamate added (ppm)	Recovered (ppm)±S.D.	Recovery (%)
3.0	–	2.98±0.10	99.33
30.0	–	29.00±0.76	96.66
–	2.0	1.97±0.09	98.70
–	20.8	19.83±0.65	95.33

the same concentrations. The precision of the method was established by analysing, six times, the same urine sample. Within-day and day-to-day assays were performed and the coefficient of variation is shown in Table 2. The specificity of the method was demonstrated by comparing the results obtained for the same amounts of cyclohexylamine in water, blank urine and urine with the addition of amino acid standard solution (A9906 from Sigma) and 6000 μM urea. The R.S.D. of the three sets of results was 1.5%.

The limit of detection in routine work was calculated by means of a signal-to-noise ratio of 3:1, and the value was found to be 0.06 ppm of cyclohexylamine corresponding to 0.11 ppm of cyclamate anion. The limit of quantitation was calculated as ten times the standard deviation in urine containing 0.10 ppm of cyclohexylamine, and this value was found to be 0.10 ppm.

The linearity of this analytical method was ascertained and was shown to lie in the range from 0.0 to 33.3 ppm of cyclohexylamine solutions containing 4.4 ppm of cycloheptylamine, with a coefficient r^2 of 0.999. The ruggedness of the method was ascertained to be very good by changing the batch of chemicals and the analyst (less than 2% of R.S.D.). Finally, the robustness was observed to be good by changing the pH of the eluent, the pH in the derivatization reaction media and the wavelength of the spectrophotometric detector. The respective results remained unaffected by variations of less than one unit of pH or 5 nm in wavelength.

Table 2
Precision of assays for cyclamate in urine

	Range±S.D. (ppm)	R.S.D. (%)
Within-day ($n=6$)	7.50±0.20	2.72
	0.18±0.01	5.55
Day-to-day ($n=6$)	7.60±0.08	1.10
	0.180±0.005	2.92

3.2. Other fields of application

Sodium cyclamate and cyclohexylamine content in sweetener sachets and in some beverages are currently being studied with the present analytical method. In the case of diet sweetener bags of general use in snack-bars, the subsequent analysis of an aqueous solution of the powder revealed a content of 6.1±0.2% if it were sodium cyclamate. The factory laboratory informed us that in the production of sweeteners they use a mixture containing 6.5% (w/w) of sodium cyclamate. Assays on cola drink (light) and soda water yielded a content of 390±4.6 and 75±0.9 ppm expressed as sodium cyclamate. No free cyclohexylamine was found in such samples.

4. Conclusions

The derivatization of cyclohexylamine in urine samples by means of TNBS, in a basic medium, appears to be a useful HPLC pre-column derivatization method to determine the content of free cyclohexylamine and cyclamate in such samples. To quantify the cyclamate content, it is necessary to oxidize the cyclamate to cyclohexylamine by using hydrogen peroxide in a hot acidic medium, followed by neutralization. A spectrophotometric UV–Vis detector, set at 335 nm, provides a reliable, sensitive, and quick method for such determinations, its limit of detection being 0.06 ppm of cyclohexylamine. No preparation of samples is needed other than centrifugation with a microcentrifuge at 12 500g. In addition, the method reported may be semi-automated if an automatic injector with a pre-column derivatization facility is acquired. Interferences with amino acids and urea do not exist and no other interferences were found. Other fields of application in clinical chemistry and in dietary food control are currently being tested.

The limit of detection (L.O.D.) and the relative standard deviation (R.S.D.) of the measurements were 0.11 ppm of cyclamate and 1.5%, respectively. These figures lie in the low zone of the methods previously described in the literature using HPLC with a UV–Vis detector: 2 ppm L.O.D. and 6.8%

R.S.D. [18], 5 ppm L.O.D. and 1.8% R.S.D. [21], 0.04 ppm and 2.6% R.S.D. [16]. Other reagents used in the literature, such as 4-fluoro-7-nitrobenzene [18] and *o*-phthalaldehyde (OPA) [16,21], yield derivatives with other substances in the sample that can interfere with the target peaks. In those cases the chromatograms are less clear, especially when low levels of cyclamate are studied.

The main advantages of the present analytical method are: (a) the simplicity of all the steps in sample preparation; (b) the stability of the N-trinitrophenyl derivatives for at least 72 h at room temperature and open atmosphere; (c) the clarity of the chromatograms; (d) the simplicity and cheapness of the chromatograph used, enabling this analysis to be performed in almost all laboratories. Finally, a further advantage is the short time per run, 14 min from one injection to another.

Acknowledgments

This work was subsidised by a grant from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad (FIS 95/1761), and by money provided under a research agreement between Productos Aditivos S.A. and the Universitat de Barcelona (contract FBG 93/1711). The help provided by Dr. Lourdes Riba Barba and Cristina Inglés González is gratefully acknowledged.

References

[1] T.R.A. Davis, N. Adler, J.C. Opsahl, Toxicol. Appl. Pharmacol., 15 (1969) 106.
[2] A.J. Collins, Diabetes Care, 12 (1989) 50.
[3] B.S. Drassar, A.G. Renwick and R.T. Williams, Biochem. J., 129 (1972) 881.
[4] B.A. Bopp, R.C. Sonders and J.W. Kesterson, Crit. Rev. Toxicol., 16 (1986) 213; A.G. Renwick, Xenobiotica, 16 (1986) 1057; A.G. Renwick, Food Addit. Contam., 8 (1991) 135.
[5] National Research Council, Report of the Ad Hoc Committee on Non-nutritive Sweeteners, Food and Nutrition Board, Academy of Sciences, Washington, USA, 1969; J.M. Price, C.G. Biava, B.L. Oser, E.E. Vogin, J. Stainfeld and H.L. Ley, Science, 167 (1970) 1131; F.Burbank and J.F. Fraumeni Jr., Nature, (1970) 227.
[6] F.E. Ahmed and D.B. Thomas, Crit. Rev. Toxicol., 22 (1992) 81; J.A. Risch, J.D. Burch, A.V. Miller, G.B. Hill, R. Still and G.R. Howe, Am. J. Epidemiol., 127 (1988) 1179; D.B. Armstrong, IARC, Sci. Publ., 65 (1985) 129; National Academy of Sciences, Evaluation of Cyclamate for Carcinogenicity, National Research Council, Committee on the Evaluation of Cyclamate for Carcinogenicity, National Academy Press, Washington, USA, 1985.
[7] B.L. Oser, S. Carson, G.E. Cox, E.E. Vogin and S.S. Sternberg, Toxicology, 4 (1975) 315; I.F. Gaunt, J. Hardy, P. Grasso, S.D. Gangoli and K.R. Butterworth, Food Cosmet. Toxicol., 14 (1976) 269; J. Hardy, I.F. Gaunt, J. Huson, R.J. Hendy and K.R. Butterworth, Food Cosmet. Toxicol., 14 (1976) 269.
[8] N.E. Buss, A.G. Renwick, K.M. Donaldson and C.F. George, Toxicol. Appl. Pharmacol., 115 (1992) 199.
[9] K. Hwang, Arch. In. Pharmacodyn., 163 (1966) 303.
[10] R.W. James, R. Haywood and D. Crook, Food Cosmet. Toxicol., 19 (1981) 291; K. Mastalski, Final report to Abbot Laboratories: Chronic Oral Toxicity Study with Cyclohexylamine Sulfate in Beagle Dogs, IBT No. 8580-07569 (651-05234), Industrial Bio-Test Laboratories, Northbrook, Il, USA, 1981; A. Roberts, A.G. Renwick, G. Ford, D. Creasy and F. Gaunt, Toxicol. Appl. Pharmacol., 98 (1989) 216.
[11] D. Brusick, M. Cifone, R. Young and S. Benson, Environ. Mol. Mutagen., 14 (1989) 189; K.T. Cain, C.W. Cornett, N.L.A. Cacheiro, L.A. Huges, J.G. Owens and W.M. Generoso, Environ. Mol. Mutagen., 11 (1988) 207.
[12] W. von Groebel and A. Wessels, Dtsch. Lebensm. Rundsch., 68 (1972) 393.
[13] D.E. Johnson and H.B. Nunn, J. Assoc. Off. Anal. Chem., 51 (1968) 1274.
[14] I.M. Psarellis, E.G. Sarantonis and A.C. Calokerinos, Anal. Chim. Acta, 272 (1993) 265.
[15] U. Zache and H. Gründing, Z. Lebensm. Unters. Forsch., 184 (1987) 503.
[16] J. Rüter and D.I.U. Racczek, Z. Lebensm. Unters. Forsch., 194 (1992) 520.
[17] M. Lehr and W. Schmid, Z. Lebensm. Unters. Forsch., 192 (1991) 335.
[18] M. Hauck and H. Köbler, Z. Lebensm. Unters. Forsch., 191 (1990) 322.
[19] T.A. Biemer, J. Chromatogr., 463 (1989) 463.
[20] J.F. Lawrence, Analyst, 112 (1987) 879.
[21] G. Schwedt and M. Hauck, Z. Lebensm. Unters. Forsch., 187 (1988) 127.
[22] A. Herrmann, E. Damawandi and M. Wagmann, J. Chromatogr., 280 (1983) 85.
[23] W.L. Caudill and R.M. Wightman, Anal. Chim. Acta, 141 (1982) 269.
[24] P.M. Kabra, P.K. Bhatnager and M.A. Nelson, J. Chromatogr., 307 (1984) 224.